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Sweet memories: hippocampal regulation of energy intake

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SWEET MEMORIES: HIPPOCAMPAL REGULATION OF ENERGY INTAKE

by

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Under the Direction of Marise B. Parent, PhD

ABSTRACT

The memory of a recently eaten meal provides a record of intake that influences future eating behavior. In humans, impairing the encoding of a meal-related memory increases the amount consumed during the next meal, whereas enhancing the memory of meal decreases the amount consumed during the next feeding bout. The brain regions that mediate these mnemonically-driven effects on energy intake are largely unknown. The aim of this dissertation is to identify whether brain regions critical for memory and the mechanisms that underlie memory formation regulate feeding behaviors. The hypothesis guiding this dissertation is that dorsal hippocampal (dHC) neurons, which are essential for episodic and spatial memory, and ventral hippocampal (vHC) neurons, which are associated with affective and emotional memory, form a meal-related memory during the postprandial period that inhibits future intake. The studies outlined herein used pharmacological and optogenetic methods to inhibit dHC

and vHC neurons before, during, or after a sucrose, standard chow and saccharin meal to determine whether and when dHC and vHC neurons are involved in regulating energy intake. The findings show that neural activity in both dHC and vHC neurons during the period following ingestion, when the memory of the meal would be undergoing consolidation, is particularly critical for limiting future intake. To determine if dHC and vHC neurons utilize mechanisms of synaptic plasticity necessary for memory formation to regulate energy intake, experiments in this dissertation also tested whether dHC and vHC regulation of intake required N-methyl-D-aspartate receptors (NMDARs) and activity-regulated cytoskeletal protein (*Arc*) expression. The results suggested that NMDARs and *Arc* in vHC but not dHC inhibit future energy intake. Collectively, these findings support the hypothesis that hippocampal neurons form meal-related memories that inhibit future intake and provide a more complete understanding of how the brain controls energy intake. Improving our understanding of how the brain regulates energy intake is essential for developing new interventions to help control diet-induced obesity.

INDEX WORDS: Hippocampus, memory, synaptic plasticity, feeding behavior, energy regulation, sucrose

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DEDICATION

To the one that said yes. Without your constant love and support this dissertation would not have been written. I love you and thank you for your endless patience.

“I am Defeated all the time, yet to Victory I am born.”

-R.W. Emerson

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LIST OF ABBREVIATIONS

AMPAR(s) – α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor(s)
Arc – Activity-regulated cytoskeletal protein
BNST– Bed nucleus of the stria terminalis
dHC – Dorsal hippocampus/al
GLP-1 – Glucagon-like peptide 1
Glu – Glutamatergic
HC – Hippocampus/al
LS – Lateral septum
LH – Lateral hypothalamus
mGluR – Metabotropic glutamate receptor
NA – Nucleus accumbens
NMDAR(s) – N-methyl-D-aspartate receptor(s)
NT-4 – Neurotrophin-4
ppIMI – postprandial intermeal interval
TNF-α – Tumor necrosis factor α
vHC – Ventral hippocampus/al
VTA – Ventral tegmental area

1 INTRODUCTION

1.1 The role of memory in energy regulation

Memory of a meal likely influences energy intake because it provides a record of recent energy intake that outlasts most physiological signals produced by eating. One of the first examples of the importance of memory in energy regulation was provided by the famous patient H.M. who had his medial temporal lobe, including the hippocampus, removed to alleviate severe epilepsy. This surgery resulted in a temporally-graded retrograde amnesia and severe anterograde amnesia (Scoville and Milner 1957, Squire 2009). H.M. was also unable to interpret internal states such as hunger, could not remember eating, and would eat when presented with food even when satiated (Hebben et al. 1985). The inability to interpret internal hunger signals, impaired memory of eating, and overconsumption has been observed in other patients that have severe memory deficits (Rozin et al. 1998, Higgs et al. 2008). In intact humans, enhancing the encoding of meal decreases the amount of food consumed during the following meal, whereas disrupting the encoding of a memory of a meal increases the amount of food that is later consumed (Robinson et al. 2013). Interestingly, enhanced encoding of a meal does not affect the size of the meal being encoded (Brunstrom and Mitchell 2006, Higgs and Woodward 2009, Oldham-Cooper et al. 2011), suggesting that the memory of a meal limits future intake and influences energy regulation during the time between two meals (i.e., during the postprandial intermeal interval [ppIMI]; Robinson et al. 2013). Moreover, the memory of how much was consumed during a recently eaten meal serves as a better predictor of how hungry one feels later than the actual amount of food ingested during that recently eaten meal (Brunstrom et al. 2012).

1.2 Meal initiation and meal size

Most studies examining the neural controls of energy intake focus on the factors that drive animals to stop eating and limit meal size (Smith 1996, Davis et al. 2000, Smith 2000, Davis et al. 2001, Smith 2001, Strubbe and Woods 2004), whereas few focus on neural controls of meal onset and the processes that control the interval between meals. Evidence suggests that endogenous signals generated by a meal influence when an animal will eat again (Kraly et al. 1978, Strubbe et al. 1986, Collier et al. 1999). Prior to meal onset, hormonal signals such as ghrelin peak (Cummings and Overduin 2007) and plasma glucose levels decrease (Louis-Sylvestre and Le Magnen 1980, Campfield et al. 1985, Smith and Campfield 1993). Circadian rhythms (Nagai et al. 1978, Kersten et al. 1980) control meal patterning and learned environmental stimuli can cause sated animals to initiate a meal (Reppucci and Petrovich 2012).

We contend, however, that cognitive controls such as memory also play a critical role in regulating energy intake. Specifically, we hypothesize that hippocampal (HC) neurons from meal-related memories that inhibit meal onset during the ppIMI. Recent work from our laboratory indicates that the period immediately following a meal may be critical for the ability of HC neurons to inhibit future intake (Henderson et al. 2013). Dorsal hippocampal (dHC) infusions of the $GABA_A$ agonist muscimol given at the end of a sucrose meal (i.e., when the memory of the sucrose meal should be undergoing consolidation) accelerates the initiation of the next meal and increases the amount eaten at the post-infusion meal (Henderson et al. 2013).

1.3 Hippocampal regulation of energy intake

The HC is a brain structure essential for learning and memory (Hunsaker et al. 2008, Kesner et al. 2008, Fanselow and Dong 2010, Barbosa et al. 2012, Strange et al. 2014). It can be functionally divided along its septo-temporal axis into the dHC (posterior in primates) and ventral hippocampus ([vHC], anterior in primates; Moser and Moser 1998, Fanselow and Dong 2010, Strange et al. 2014). dHC neurons are necessary for episodic and spatial memory, whereas vHC neurons are essential for affective memory and motivational processes (Fanselow and Dong 2010, Strange et al. 2014). Memories are mediated by multiple brain areas that each represent different information contained within the same experience (White et al. 2013, Gasbarri et al. 2014). Meal-related memories may contain both episodic-like and affective components, raising the possibility that dHC and vHC neurons encode memories for different aspects of a meal and that their combined efforts may contribute to the formation of meal-related memories that inhibit future intake.

Neuroanatomical evidence suggests that both dHC and vHC neurons are poised to integrate food-related signals with mnemonic processes. HC neurons express receptors for several food-related signals, such as leptin (Mercer et al. 1996), ghrelin (Noble et al. 1999), gastrin-releasing peptide (a.k.a. bombesin; Kamichi et al. 2005), melanocortins (Mul et al. 2013, Shen et al. 2013), neuropeptide-Y (Dumont et al. 1993), cholecystokinin (Zarbin et al. 1983), and glucagon-like peptide-1 (GLP-1; During et al. 2003). HC neurons also receive external sensory information and interoceptive cues about food from the vagus nerve (Wang et al. 2006), locus coeruleus (Wyss et al. 1979), dorsal raphe nuclei (Wyss et al. 1979), and VTA (Gasbarri et al. 1994, Gasbarri

et al. 1994; reviewed in Kanoski and Grill 2015). vHC neurons are the primary source of HC projections to downstream brain regions critical for energy intake: the bed nucleus of the stria terminalis (BNST; Radley and Sawchenko 2011), lateral septum (LS; Risold and Swanson 1996, Risold and Swanson 1997), nucleus accumbens (NA; Namura et al. 1994), prefrontal cortex (Hsu et al. 2017), and lateral hypothalamus (Cenquizca and Swanson 2006, Hsu et al. 2015). dHC and vHC neurons can communicate with each other through bidirectional, intra-HC projections (Amaral and Witter 1989, Ishizuka et al. 1990, Andersen 2007, Strange et al. 2014).

Evidence suggests that direct manipulation of HC neurons and their projections influences energy intake. Gross lesions of the HC increase meal frequency (Clifton et al. 1998), and lesions specific to vHC increase body weight and total food consumption (Davidson et al. 2009). vHC infusions of the orexigenic hormone ghrelin increase food intake (Kanoski et al. 2013) and activation of vHC receptors for gut hormones GLP-1 and leptin reduces food intake (Kanoski et al. 2011, Hsu et al. 2015). vHC infusions of ghrelin, leptin, and GLP-1 also enhance food-related memory (Kanoski et al. 2011, Kanoski et al. 2013, Hsu et al. 2015). Moreover, optogenetic activation of glutamatergic vHC projections to either LS (Sweeney and Yang 2015), BNST (Sweeney and Yang 2015), or prefrontal cortex reduce energy intake (Hsu et al. 2017).

1.4 Synaptic plasticity

One of the principal mechanisms for the long-term storage of information in the HC is the modification of synaptic efficacy between neurons (i.e., synaptic plasticity). Activation of glutamatergic N-methyl-D-aspartate receptors (NMDARs) is required for most forms of synaptic plasticity in the HC (Malenka and Nicoll 1993, Li and Tsien 2009,

Luscher and Malenka 2012). Activation of NMDARs increases intracellular Ca^{++} levels and initiates a series of downstream molecular cascades resulting in the transcription of new mRNA and the local synthesis of several protein products aimed at increasing AMPA receptor activity (Davis et al. 1992, Cammarota et al. 2000, Bast et al. 2005, Bevilaqua et al. 2005, Bloomer et al. 2008, Bourne et al. 2013). Active synapses utilize new protein products to increase AMPA receptor distribution at post-synaptic density zones (Chowdhury et al. 2006), alter receptor type subunits (Plant et al. 2006), and the size and type of dendritic spines (Peebles et al. 2010). These changes that enhance synaptic strength within the HC also increase functional connectivity with downstream brain regions (Canals et al. 2009).

Pharmacological blockade of HC NMDARs inhibits synaptic plasticity and disrupts memory formation (Czerniawski et al. 2011, Czerniawski et al. 2012, Park et al. 2014). Feeding related signals such as insulin (Liu et al. 1995), ghrelin (Muniz and Isokawa 2015) and leptin (Shanley et al. 2001) converge on NMDARs amplifying NMDAR-mediated currents. This amplification may be critical for integrating feeding related signals into meal-related memories. *Whether HC neurons also inhibit energy intake through an NMDAR-dependent process is unknown*.

1.5 Activity-regulated cytoskeletal-protein (*Arc*)

Numerous molecular cascades contribute to synaptic plasticity and several of these pathways converge onto the immediate early gene *Arc* (Waltereit et al. 2001, Ying et al. 2002). *Arc* is considered a master regulator of synaptic plasticity (Bramham et al. 2010, Korb and Finkbeiner 2011, Shepherd and Bear 2011). Learning experiences rapidly induce *Arc* expression (Guzowski et al. 2001, Kelly and Deadwyler 2002,

Ramirez-Amaya et al. 2005, Vazdarjanova et al. 2006, Miyashita et al. 2009) and *Arc* is transported to the dendrites of recently activated synapses (Steward et al. 1998) in an NMDAR-dependent manner (Steward and Worley 2001). Once at the dendrite, *Arc* is an effector molecule that interacts with cytoskeletal proteins and increases AMPA receptor trafficking (Chowdhury et al. 2006). *Arc* is necessary for long-term memory formation in both dHC and vHC-dependent memory tasks (Guzowski et al. 2000, Plath et al. 2006, Bramham et al. 2010, Czerniawski et al. 2011, Shepherd and Bear 2011, Czerniawski et al. 2012, Chia and Otto 2013). *Arc* is required for the consolidation of memory, but not for the initial learning of a behavior (Plath et al. 2006). Our laboratory found that sucrose consumption increases *Arc* expression in dHC neurons during the postprandial period (Henderson et al. 2016), suggesting that *Arc* may be critical for regulating future intake during the ppIMI when a meal-related memory should be undergoing consolidation. *It is unknown whether Arc is necessary for HC regulation of energy intake.*

1.6 Neurotrophin-4 (*ntf4*)

Synaptic plasticity involves several molecular pathways. To determine which genes critical for synaptic plasticity also regulate hippocampal-dependent energy intake, our lab used a qRT-PCR approach to examine the simultaneous expression of 84 plasticity-associated genes in both dHC and vHC neurons 30 min after sucrose consumption. One notable finding was that sucrose consumption increased the expression of the *ntf4* gene 15-fold in dHC neurons and decreased *ntf4* expression ~4-fold in vHC neurons. The increase of dHC *ntf4* expression was the largest of all of the genes that were measured.

Little is known about the role of n*tf4* in synaptic plasticity. *Ntf4* encodes NT-4, a

ligand of the TrkB receptor (Barbacid 1995). Some forms of hippocampal-dependent memory increase *ntf4* in the dentate gyrus of the HC (Callaghan and Kelly 2013). *Ntf4* also increases phosphorylation of glutamatergic AMPARs and enhances HC synaptic plasticity (Zeng et al. 2010). Deletion of *ntf4* gene inhibits long-term memory and decreases long-lasting long-term potentiation (Fan et al. 2000, Xie et al. 2000). *It is unknown whether dHC ntf4 regulates energy intake*.

1.7 Dissertation Aims

There is a preponderance of research implicating homeostatic and hedonic processes in the neural control of energy regulation (reviewed in Lutter and Nestler 2009, Johnson 2013), whereas few studies have examined the role of cognition in regulating ingestive behavior (Parent et al. 2014, Parent 2016). Cognitive processes such as memory may serve as an important control of future consumption because they provide a record of recent intake. The HC is essential for mnemonic processing: vHC neurons are important for emotional memory and motivational processes, whereas dHC neurons are essential for episodic memories (reviewed in Fanselow and Dong 2010, Strange et al. 2014). Meal-related memories may involve both emotional (i.e., how the meal made one feel) and episodic (i.e., the what, the when, and how much one ate) components. Changes in synaptic efficacy are hypothesized to be the main neural mechanisms underlying memory formation and storage (reviewed in Takeuchi et al. 2014). Acquisition and consolidation of HC-dependent memory typically requires N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity at glutamatergic synapses (Luscher and Malenka 2012) and the NMDA-dependent expression of *Arc*. **We hypothesize that consumption of a meal induces NMDAR-dependent synaptic**

plasticity in HC neurons during the postprandial period and that this synaptic plasticity is necessary for HC inhibition of energy intake during the time between two meals.

Aim 1: Determine whether vHC neurons inhibit energy intake during the ppIMI and whether NMDAR-dependent synaptic plasticity is necessary.

Although evidence indicates that vHC neurons influence eating behavior and body weight (Davidson et al. 2009, Kanoski et al. 2011, Hsu et al. 2015), it is unknown whether vHC neurons inhibit intake during the ppIMI and whether vHC control of intake requires NMDAR-dependent synaptic plasticity. We predicted that pharmacological inhibition of vHC neurons with vHC muscimol infusions given after a sucrose meal accelerates the onset of the next meal and increases the total amount that is subsequently ingested. We also predicted that sucrose ingestion increases *Arc* expression and that infusions of a vHC NMDAR antagonist or *Arc* antisense oligodeoxynucleotides decrease the ppIMI and increases energy intake.

Aim 2: Determine when dHC and vHC glutamatergic neurons are critical for the regulation of energy intake.

Pharmacological infusions of muscimol inhibit neural activity for several hours (Martin 1991, Arikan et al. 2002) and thus cannot be restricted to the memory consolidation period. Postmeal muscimol infusions likely suppress neuronal activity during the entire ppIMI and during consumption of the next meal. It is therefore impossible to determine whether the muscimol-induced increase in consumption that is observed following postmeal infusions is the result of disrupted processes during the ppIMI and/or due to neuronal inhibition during the consumption of the next meal.

Moreover, it is unclear whether dHC or vHC neurons also influence feeding before or during a meal and whether they influence intake through other memory processes, such as encoding and retrieval of meal-related memories. We predicted that optogenetic inhibition of dHC or vHC glutamatergic neurons given for 10 min after a meal has ended decreases the ppIMI and increases intake during the next meal and that inhibition given before or during a meal will be less effective. To determine whether dHC and vHC neurons only control intake of palatable foods, we tested whether optogenetic inhibition of these neurons also increases intake of home cage diet. Given that HC damage impairs the ability to interpret visceral interoceptive signals (Hebben et al. 1985, Rozin et al. 1998, Higgs et al. 2008), we also determined whether optogenetic inhibition of dHC and vHC glutamatergic neurons increases saccharin intake as saccharin intake is controlled primarily by orosensation rather than interoceptive, postingestive signals (Byard and Goldberg 1973, Mook et al. 1980, Mook et al. 1981, Kushner and Mook 1984, Renwick 1985, Sclafani and Nissenbaum 1985, Renwick 1986).

Aim 3: Determine whether dHC *Arc* and *ntf4* inhibit energy intake and influence body weight.

Our research group showed previously that consumption of a sucrose meal increases two biomarkers of synaptic plasticity in dHC: *Arc* mRNA (Henderson et al. 2016) and *ntf4* mRNA (unpublished), and that muscimol-induced inactivation of dHC neurons following a sucrose meal accelerates the onset of the next meal and increases intake during the next meal (Henderson et al. 2013). It is not known, though, whether dHC neurons inhibit energy intake through a process that requires NMDARs, *Arc,* and *ntf4*. We predicted that pharmacologically blocking NMDARs or using RNA interference

(RNAi) strategies to chronically disrupt dHC *Arc* or *ntf4* expression increases acute sucrose consumption, total food intake, and body weight.

The research described in this dissertation demonstrates a novel role for brain structures involved in memory and implicates synaptic plasticity in the regulation of energy intake, particularly meal frequency and meal size. Specifically, this dissertation investigates how neural activity in HC glutamatergic neurons during the ppIMI limits future intake and identifies a novel role for vHC and dHC neurons in regulating ingestive behaviors and implicates synaptic plasticity in HC control of energy intake. Investigating how synaptic plasticity contributes to HC regulation implicates the mechanisms that underlie learning and memory in energy regulation.

Improving our understanding of how the brain regulates energy intake is essential for developing new interventions to help control diet-induced obesity. Worldwide obesity rates have more than doubled in the last 20 years (Ogden et al. 2014). Almost 39% of the world population is considered overweight, with 13% of adults being characterized as obese (Ogden et al. 2014). The severe increase in body mass is due, in large part, to excessive intake of energy typically brought on by a decrease in the interval between eating episodes (i.e., snacking; Nielsen et al. 2002, Cutler et al. 2003, Nicklas et al. 2003), increased consumption of high energy foods (Mozaffarian et al. 2011, Monteiro et al. 2013, Moubarac et al. 2013, Slining et al. 2013), and a reduction in overall activity levels (Cutler et al. 2003, Janiszewski and Ross 2007, Bleich et al. 2008). These increases in body mass and obesity decrease HC activity during the encoding of memories (Cheke et al. 2017), impair episodic memory (Cheke et al. 2016, Prickett et al. 2018), and are correlated with HC atrophy (Shefer et al. 2013, Bauer et al.

2014, Cherbuin et al. 2015, Jacka et al. 2015). Weight loss and body mass reduction, however, can improve memory function (Miller et al. 2013, Alosco et al. 2014) and increasing the memory of a recently eaten meal reduces energy intake (Robinson et al. 2013, Robinson et al. 2014).

The research outlined in this dissertation reveals when dHC and vHC neurons are most critical for regulating energy intake and the mechanisms for HC control of food consumption. A more complete understanding of how the brain inhibits eating may lead to the development of novel cognitive or pharmacologically-based interventions to reduce energy intake and treat eating-related disorders.

2 VENTRAL HIPPOCAMPAL NEURONS INHIBIT POSTPRANDIAL ENERGY INTAKE

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Key words: activity regulated cytoskeletal protein, memory, muscimol, plasticity, sucrose

2.1 Abstract

Evidence suggests that the memory of a recently ingested meal limits subsequent intake. Given that ventral hippocampal (vHC) neurons are involved in memory and energy intake, the present experiment tested the hypothesis that vHC neurons contribute to the formation of a memory of a meal and inhibit energy intake during the postprandial period. We tested 1) whether pharmacological inactivation of vHC neurons during the period following a sucrose meal, when the memory of the meal would be undergoing consolidation, accelerates the onset of the next sucrose meal and increases intake and 2) whether sucrose intake increases vHC expression of the synaptic plasticity marker activity-regulated cytoskeletal-associated protein (*Arc*). Adult male Sprague-Dawley rats were trained to consume a 32% sucrose solution daily at the same time and location. On the experimental day, the rats were given intra-vHC infusions of the $GABA_A$ receptor agonist muscimol or vehicle after they finished their first sucrose meal. Compared to vehicle infusions, postmeal intra-vHC muscimol infusions decreased the latency to the next sucrose meal, increased the amount of sucrose consumed during that meal, increased the total number of sucrose meals and the total amount of sucrose ingested. In addition, rats that consumed sucrose had higher levels of *Arc* expression in both vHC CA1 and CA3 subfields than cage control rats. Collectively, these findings are the first to show that vHC neurons inhibit energy intake during the postprandial period and support the hypothesis that vHC neurons form a memory of a meal and inhibit subsequent intake.

2.2 Introduction

Decades of research on the neural controls of ingestive behavior has focused primarily on the role of homeostatic and hedonic processes; by contrast, there is a limited understanding of how brain areas involved in cognition inhibit energy intake. Investigating how brain areas involved in cognition limit eating will provide a more complete explanation of the neural mechanisms that control ingestion and may enhance our understanding of eating-related disorders such as obesity. Research in humans suggests that the encoding and maintenance of an episodic meal-related memory limits future energy intake. For instance, enhancing the memory of a meal decreases the amount of food consumed during the subsequent eating episode (Robinson et al., 2013a), whereas distraction during a meal results in greater feelings of hunger and increased consumption during the next bout (Brunstrom and Mitchell, 2006; Higgs and Woodward, 2009; Oldham-Cooper et al., 2011). In addition, the memory of how much was consumed in a recently eaten meal is a better predictor of future hunger than the actual amount of food ingested (Brunstrom et al., 2012). Interestingly, the famous patient H.M. and others with severe episodic memory deficits do not remember having just eaten and will eat an additional meal if presented with food after eating to satiation (Hebben et al., 1985; Rozin et al., 1998; Higgs et al., 2008). Importantly, paying attention to the food that is being consumed during a meal does not affect the size of that meal, but does decrease the amount consumed during the next eating bout in obese patients (Robinson et al., 2014).

The hippocampus is a brain structure essential for episodic learning and memory (Hunsaker et al., 2008; Kesner et al., 2008; Fanselow and Dong, 2010; Barbosa et

al., 2012; Strange et al., 2014). Neuroanatomical evidence suggests that the hippocampus is also poised to integrate food-related signals with mnemonic processing. Hippocampal neurons express receptors for several food-related signals, such as leptin (Mercer et al., 1996), ghrelin, gastrin-releasing peptide (a.k.a. bombesin; Kamichi et al., 2005), melanocortins (Mul et al., 2013; Shen et al., 2013), neuropeptide-Y (Dumont et al., 1993), and cholecystokinin (Zarbin et al., 1983). Moreover, hippocampal neurons receive neural signals from multiple brain areas that communicate external sensory information and interoceptive cues about food (reviewed in Kanoski and Grill, 2015). In turn, hippocampal neurons project to several brain regions critical for food intake, such as the lateral hypothalamus (LH; Cenquizca and Swanson, 2006; Hsu et al., 2015b), bed nucleus of the stria terminalis (BSNT; Radley and Sawchenko, 2011), lateral septum (LS; Risold and Swanson, 1996, 1997), and nucleus accumbens (NA; Namura et al., 1994).

The hippocampus can be functionally divided along its longitudinal axis into dorsal (posterior in primates) and ventral (anterior in primates) poles (Moser and Moser, 1998; Fanselow and Dong, 2010; Strange et al., 2014). Generally, dorsal hippocampal (dHC) neurons are necessary for episodic and spatial memory, whereas ventral hippocampal (vHC) neurons are essential for affective and motivational processes and emotional memory (Fanselow and Dong, 2010; Strange et al., 2014). Thus, both areas potentially contribute to the memory of eating episodes. In support, evidence suggests that both dHC and vHC are involved in energy regulation. For instance, we found previously that pharmacological inhibition of dHC neurons with infusions of the $GABA_A$ agonist muscimol given during the period following a sucrose

meal (i.e., the postprandial intermeal interval [ppIMI]; when the memory of the preceding meal would be expected to be undergoing consolidation) accelerates the onset of the next sucrose meal and increases the amount consumed during the next meal (Henderson et al., 2013). We also found that consuming sucrose increases the expression of the immediate early gene activity-regulated cytoskeleton-associated-protein (*Arc*) in dHC neurons, suggesting that sucrose ingestion induces synaptic plasticity in dHC (Henderson et al., 2016). This interpretation is supported by previous research showing that a learning experience elevates hippocampal *Arc* expression and that these increases in *Arc* are necessary for memory consolidation in both dHC- and vHC-dependent memory tasks (Guzowski et al., 2000; Plath et al., 2006; Bramham et al., 2010; Czerniawski et al., 2011; Shepherd and Bear, 2011; Czerniawski et al., 2012; Chia and Otto, 2013). Moreover, *Arc* expression is not a measure of neural activity (Fletcher et al., 2006; Guzowski et al., 2006); rather, the amount of *Arc* expressed is correlated with electrophysiological measures of synaptic plasticity (Carpenter-Hyland et al., 2010). Collectively, our findings support the hypothesis that dHC neurons form a memory of a meal and reduce future intake.

Several lines of evidence have led us to hypothesize that vHC neurons also participate in forming a memory of an eating episode and inhibit energy intake during the period following intake (i.e., the ppIMI). Specifically, previous findings have shown that vHC lesions increase body weight and food consumption (Davidson et al., 2009) and that vHC infusions of the orexigenic hormone ghrelin increase food intake (Kanoski et al., 2013). In addition, activation of receptors for the gut hormones glucagon-like peptide-1 (GLP-1) and leptin in vHC reduces food intake (Kanoski et al., 2011; Hsu et

al., 2015a), and vHC infusions of ghrelin, GLP-1, and leptin also affect food-related memory (Kanoski et al., 2011; Kanoski et al., 2013; Hsu et al., 2015a). Moreover, optogenetic activation of specific vHC projections to either BNST or LS markedly reduces total food consumption; whereas, chemogenetic inactivation of the dentate gyrus-CA3 region of vHC increases energy intake (Sweeney and Yang, 2015). Importantly, vHC neurons are the primary source of hippocampal projections to brain regions critically involved in energy regulation such as LH, BNST, LS, and NA (Walaas and Fonnum, 1980; Kishi et al., 2000; Cenquizca and Swanson, 2006; Hsu et al., 2015b).

Combined, these studies show that vHC neurons influence energy regulation; however, it is unknown whether vHC neurons regulate energy intake during the ppIMI when the memory of the meal is likely undergoing consolidation. As a result, the present study determined whether temporary inactivation of vHC neurons with infusions of muscimol given at the end of a sucrose meal accelerates the onset of the next meal and increases intake. If vHC neurons influence future intake through a process that involves memory, then ingestion should induce synaptic plasticity in these neurons. As a molecular marker of plasticity, we also determined whether sucrose consumption increases *Arc* expression in vHC neurons.

2.3 Materials and Methods

2.3.1 *Subjects*

Adult male Sprague-Dawley rats (postnatal day 52–58 on arrival; Charles River Laboratories, Wilmington, MA) were individually housed in Optirat® cages (Animal Care Systems, Centennial, CO). The rats were placed on a 12:12 light/dark cycle and

given *ad libitum* access to pelleted food and water in their home cages. The Georgia State University Institutional Animal Care and Use Committee approved all procedures.

2.3.2 *Surgery*

At least 7 days after arrival, the rats were anesthetized with 5% isoflurane (Henry Schein Impromed, Oshkosh, WI) in 1,000 ml/min of oxygen (Airgas, Radnor, PA) and given penicillin (1,500 IU, im; Henry Schein Impromed, Oshkosh, WI) and flunixin meglumine (2.5 mg/kg, sc; Henry Schein Impromed, Oshkosh, WI). Anesthesia was maintained with 1–3% isoflurane gas in 500 ml/min oxygen for the duration of the surgery. Unilateral guide cannulae (8.5 mm long, 26-gauge; Plastics One, Roanoke, VA) were implanted aimed at the left or right vHC in a counterbalanced manner (AP: −5.3 mm, ML: +5.1mm, DV: −6.4 mm from skull (Paxinos and Watson, 1998). Previous research targeting vHC with these coordinates has implicated vHC and *Arc* in memory (Bast et al., 2001; Zhang et al., 2001; Czerniawski et al., 2011; Czerniawski et al., 2012; Chia and Otto, 2013; Zhang et al., 2014). Guide cannulae were held in place by jewelers' screws (Plastics One, Roanoke, VA) and cranioplastic cement (DuraLay, Reliance Dental Mfg. Co., Worth, IL) and an obdurator (Plastics One, Roanoke, VA) was inserted into the cannula. The rats were given 0.9% sterile saline (3.0 cc, sc; Hospira, Lake Forest, IL) at the end of surgery and allowed to recover for at least 1 week before behavioral training.

2.3.3 *Experiment 1 – Sucrose Training*

Rats (n = 15) were trained to consume a 32% sucrose solution at a scheduled time and location daily to minimize the contributions of novelty, time and contextual processes to sucrose intake. On training days, the rats were placed into designated

polycarbonate experimental cages (22 cm × 43 cm × 22 cm) in a testing room at the beginning of the light cycle. Experimental cages were equipped with a modified lickometer system that measured the change in system resistance when a rat licked from a sipper tube (Model 86062, Lafayette Instruments, Lafayette, IN). The Activity Wheel Monitoring Program (Lafayette Instruments, Lafayette, IN) recorded all sipper tube contacts. An experimenter measured latency to the first tube sipper tube contact using a Precision Solid State Time-It stopwatch (Petroleum Analyzer Company, L.P., Houston, TX).

On the first day of training, the rats were placed in the experimental cages for 8 hr without access to food or water and then given a 32% sucrose solution for 10 min. The sucrose was then removed and the rats were returned to their home cages in the vivarium 1 hr later. This constituted one training trial. The same protocol was used on the second day of training, with the exception that the rats were allowed *ad libitum* access to water during the 8-hr period. Water was not given on the first training day to increase the probability that the rats would consume the sucrose, but was then given on all training days to minimize the contributions of thirst. Each subsequent training day continued in this manner with the exception that sucrose was given after 3 hr rather than 8 hr. The 8-hr interval was used initially to increase the likelihood that the rats would approach the bottle and then decreased to 3 hr to be within the range of an average ppIMI (Snowdon, 1969). The rats were given daily training trials until their latency to consume from the sipper tube was less than 30 sec for 3 consecutive days. Once this criterion was met, the rats were given an additional training day in which they

were given access to the 32% sucrose solution for 4.5 hr to parallel sucrose availability on experimental days.

Experimental days occurred 1 to 6 days after the last training day. Rats were placed in their experimental cages in the behavior room without food for 3 hr and then given a 32% sucrose solution. An experimenter observed each rat and used a precision timer (Petroleum Analyzer Company, L.P., Houston, TX) to determine when a meal ended, which was operationally defined as 5 consecutive minutes without any sipper tube contact. This criterion was used because after 5 min without consumption there is a very low probability that a rat will initiate eating again (Zorrilla et al., 2005; Fekete et al., 2007); moreover, this 5-min criterion is associated with an increase in grooming, sniffing, rearing and resting behavior known as the behavioral satiety sequence (Antin et al., 1975; Thaw et al., 1998; Zorrilla et al., 2005). After the 5 min, the rats were removed from the experimental cages and given an intra-vHC injection of vehicle (1.0 µL; phosphate-buffered saline, [PBS]; 0.25 µL/min, Cellgro, Manassas, VA) or muscimol (0.5 µg/µl; 1.0 µL; Sigma-Aldrich, St. Louis, MO). The injection needle extended 1.0 mm beyond the bottom of the guide cannula and was left in place for 2 min following the injection to facilitate diffusion. The rats were then returned to the experimental cage for 4 hr during which sucrose intake was recorded. A within-subject design was used wherein rats were given infusions of muscimol and vehicle in a counterbalanced order with 72 hr between infusions.

After the completion of the behavioral tests, the rats were deeply anesthetized using 5% isoflurane gas (Henry Schein Impromed, Oshkosh, WI) in 1,000 ml/min of oxygen (Airgas, Atlanta, GA), decapitated and their brains removed and stored in

formalin for at least 48 hr. Brains were then sectioned (60 µm) and stained with thionin. Two observers examined the sections to estimate the infusion location 1.0 mm below the bottom of the cannula tract. In those instances when the two observers' estimates did not agree, a third opinion was solicited.

2.3.4 *Data Acquisition*

The number of licks and sipper tube contact duration were binned every 6 sec and stored in a .csv file. Meals were defined as sipper tube contact consisting of at least 30 licks followed by a period of 5 min without any sipper tube contact (Smith, 2000). The amount of time between the first and last lick was used to measure the duration of a meal, whereas meal size was estimated by measuring the total amount of time spent in contact with the sipper tube during the meal, which excluded time spent not licking. Rats that did not consume a postinfusion meal were given a maximum ppIMI of 14,400 sec (4 hr). To control for the effects of variations in the size of the preinfusion meal on the duration of the subsequent ppIMI, the satiety ratio was also used to estimate the ppIMI duration. The satiety ratio is an index of the amount of time spent not eating that is produced by the previous meal (Panksepp, 1973; Zorrilla et al., 2005). It was calculated in the present experiment by dividing the ppIMI by the size of the preinfusion meal in seconds. Total sucrose intake was estimated by weighing the bottle before and at the end of the experimental session. Experimenters were blind to treatment condition during the drug injections, behavioral scoring and histological assessment of cannula placement.

2.3.5 *Experiment 2 – Arc mRNA Expression*

Arc expression in vHC neurons was quantified in brain sections obtained in our previous study showing that sucrose consumption increases *Arc* expression in dHC neurons (Henderson et al., 2016). Rats in that previous study ($n = 6$) were trained to consume sucrose using a slightly different procedure. The rats were given the 32% sucrose solution for 30 min rather than 10 min and were given 4 days of training with the 8-hr interval rather than 2 days. Cage control rats ($n = 2$) were also placed into experimental cages and brought to the behavioral testing room, but food and chow were not removed and they were never given the sucrose solution. Starting on the 6th training day, the experimental rats were trained in the same manner with the exception that the sucrose was given after 3 hr rather than 8 hr. Rats in this experiment were trained daily for a total of 10 days (5 days of 8-hr without food prior to sucrose and then 5 days of 3-hr without food).

The experimental day occurred at least 24 hr after the last training day. The same procedures were used as in training, with the exception that the rats were given the 32% sucrose solution for 7 min. Then, they were removed from the experimental cages and anesthetized in a plastic gas induction chamber with 5% isoflurane gas (Baxter International, Deerfield, IL) in 1,000 mL/min of oxygen (Airgas, Radnor, PA) until they lost their righting reflex (~< 1 min). They were then decapitated using a guillotine and their brains were harvested rapidly, flash frozen in chilled 2-methylbutane (Thermo Fisher Scientific, Waltham, MA), and then stored at −80°C. The meal was terminated after 7 min to maximize the ability to detect intra-nuclear *Arc* foci that were specifically activated from consuming the solution (Vazdarjanova et al., 2002). For cage control

rats, food was removed for 3 hr and 7 min and then they were euthanized and their brains processed in the same manner. Control rats that consumed water were not included as this would require that rats be water-deprived, which would confound thirst-related processes.

2.3.6 *Fluorescence In Situ Hybridization*

Right hemispheres were blocked in freezing media such that at least one brain from each condition was in a block and a cryostat was used to obtain 20 µm coronal sections from each block. The sections were mounted onto glass slides and stored at −20°C until fluorescence *in situ* hybridization (FISH) procedures were performed. Only one hemisphere was chosen given that the effects of inactivation of left versus right dHC or vHC on sucrose intake did not differ (data not shown). For the FISH procedures, the tissue was fixed in 4% paraformaldehyde and then permeabilized in a 1:1 solution of acetone and methanol. Following a pre-hybridization step, a full-length digoxigenin-labeled *Arc* antisense riboprobe was applied and hybridized overnight at 56°C. After quenching of peroxidase activity, the digoxigenin tag was revealed with peroxidase-conjugated anti-digoxigenin antibody (Roche, Indianapolis, IN) and a tyramide amplification reaction using SuperGlo™ fluorescein (Fluorescent Solutions, Augusta, GA). The riboprobes were generated using MAXIscript® (Ambion, Austin, TX) *in vitro* transcription kits and digoxigenin-labeled UTP (Roche). Nuclei were counterstained with DAPI.

2.3.7 *Image Acquisition and Stereological Analysis*

Image stacks from vHC (4.8–6.4mm posterior to bregma) were collected from each animal using a 20x objective on a Zeiss AxioImager/Apotome system (Carl Zeiss,

Dublin, CA). Unbiased stereological cell counting and classification were performed as follows: (1) neuron-like cells in vHC CA1 (vCA1) and vHC CA3 (vCA3) in each image were segmented using an optical dissector method (West, 1999) and (2) segmented neurons were classified using Zeiss AxioVision imaging software (Carl Zeiss, Dublin, CA). These hippocampal subfields were chosen because they project to brain areas critical for energy intake (Risold and Swanson, 1997; Cenquizca and Swanson, 2006; Sweeney and Yang, 2015). Putative glial cells, which are those with small, intensely, and uniformly stained nuclei, were excluded from the analysis. Cells were classified as *Arc*-positive if they contained foci of transcription for *Arc*, defined as bright fluorescent foci present on at least three consecutive planes. Cells without any foci were classified as *Arc* negative. The *Arc*-positive neurons were reported as a percentage of the total number of neurons. The experimenter was blind to treatment condition during the cell counting and classification.

2.3.8 *Statistical Analyses*

All dependent variables were analyzed for normality using a Shapiro-Wilkes test. In Experiment 1, all dependent measures were non-normally distributed; therefore, Wilcoxon signed-rank tests were used to compare the effects of vehicle and muscimol infusions on meal size, duration, licking speed, and number of meals. Wilcoxon signed-rank tests with a Bonferroni correction ($P < 0.01$) were used to compare the effects of vehicle versus muscimol on sucrose consumption at each time-point across the experimental recording period. For Experiment 2, independent samples one-tailed *t*-tests were used to test the prediction that the percentage of *Arc*-expressing neurons in vCA1 and vCA3 would be higher in rats that consumed 32% sucrose than in cage

control rats. The alpha level was not corrected for these *t*-tests because it is not necessary to adjust the familywise Type 1 error rate with *a priori* planned comparisons (i.e., when the groups were predicted to be different in advance (Sheskin, 2007). In addition, a paired-samples *t*-test was used to compare *Arc* expression in vCA1 neurons to vCA3 expression in rats that consumed sucrose. Results were considered statistically significant when α values were less than 0.05. All data were analyzed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corporation, Armonk, NY) or GraphPad Prism for Windows, Version 6.0 (GraphPad Software, La Jolla, CA).

2.4 Results

2.4.1 *Experiment 1- Postmeal Inactivation of vHC Neurons Decreases the ppIMI, Increases Meal Size, Meal Frequency, and Total Intake*

Experiment 1 tested whether postmeal inactivation of vHC neurons would decrease the duration of the ppIMI and increase intake. Figure 1 depicts the location of the infusion sites in vHC. As expected, meal size (VEH M(+/−SEM) = 140.8(35.0); MUS M(+/−SEM) =103.6(19.5), $z = -0.682$, $P = 0.495$), duration (VEH M(+/−SEM) = 333.5(55.5); MUS M(+/−SEM) =295.7(51.3), $z = -0.511$, $P = 0.609$), and licking speed (VEH M(+/−SEM) = 3.5(0.2); MUS M(+/−SEM) =3.5(0.2), $z = -0.625$, $P = 0.532$) did not differ between vehicle and muscimol conditions during the preinfusion meal. Importantly, the findings indicated that vHC infusions of muscimol administered after the end of the first sucrose meal accelerated the onset of the next sucrose meal and increased the amount eaten during the postinfusion meal. Specifically, the muscimol infusions decreased the latency to the next sucrose meal (i.e., the ppIMI) ($z = -3.124$, $P = 0.002$; Fig. 2A) and the satiety ratio

($z = -2.840$, $P = 0.005$; Fig. 2B) and increased the size of the postinfusion meal ($z = -3.111$, $P = 0.002$; Fig. 2C] and licking speed [$z = -2.897$, $P = 0.004$; Fig. 2D).

The results showed further that the postmeal vHC muscimol infusions increased intake for the first 2 hr of the experimental period. Specifically, compared to vehicle infusions, the vHC muscimol infusions increased the number of meals consumed ($z = -2.994$, $P = 0.003$; Fig. 3A). Muscimol increased sucrose consumption for the first 30 min post infusion ($z = -3.238$, $P = 0.001$; Fig. 3B) and 61–120 min postinfusion ($z = -2.845$, $P = 0.004$). The effects of muscimol were not statistically significant at the other time-points with the Bonferroni correction [31–60 min: ($z = -2.510$, $P = 0.012$), 121–180 min: ($z = -2.349$, $P = 0.019$), 181–240 min: ($z = -1.742$, $P = 0.081$)]. Muscimol significantly increased the total amount of sucrose ingested ($z = -2.869$, $P = 0.004$; Fig. 3C).

2.4.2 *Experiment 2 - Sucrose Consumption Increases the Expression of the Synaptic Plasticity Marker Arc in vHC Neurons*

Consuming a 32% sucrose solution for 7 min increased *Arc* expression in both vCA1 and vCA3 neurons. Specifically, the percentage of *Arc*-expressing neurons in vCA1 ($t(6) = 6.26$, $P = 0.001$; Fig. 4) and vCA3 ($t(6) = 4.07$, $P = 0.007$; Fig. 4) was significantly higher in rats that had just consumed a 32% sucrose solution than in cage control rats. Moreover, sucrose consumption produced a bigger increase in *Arc* expression in vCA1 neurons than in vCA3 neurons ($t(5) = 4.87$, $P = 0.005$; Fig. 4).

2.5 Discussion

The present findings are the first to show that inactivation of vHC neurons during the period following a sucrose meal (i.e., during the ppIMI) accelerates the onset of the next sucrose meal and increases the amount consumed during that postinfusion meal, increases sucrose meal frequency, and the total amount of sucrose ingested. Furthermore, the present findings also indicate that sucrose consumption is associated with increased *Arc* expression in vCA1 and vCA3 neurons. Collectively, the present findings suggest that ingestion induces synaptic plasticity in vHC neurons during the postprandial period and that vHC neural activity during this period is critical for limiting future intake. These findings are consistent with our overarching hypothesis that vHC neurons contribute to the memory of a meal and inhibit meal initiation and total intake during the postprandial period. Moreover, these results add to the accumulating evidence that hippocampal neurons are a critical component of the neural circuitry that regulates energy intake.

Memory of a previous meal provides a record of recent intake that can outlast the physiological signals produced by a meal (Brunstrom et al., 2012). We speculate that eating-induced synaptic plasticity and memory inhibit intake by increasing the ability of principal hippocampal glutamatergic projection neurons to modulate neural activity in brain regions involved in energy intake. This possibility is supported by findings showing that synaptic plasticity and increased synaptic strength in hippocampus augments functional connectivity between the hippocampus and other brain regions (Canals et al., 2009), and importantly, by results showing that activation of vHC glutamatergic projections to LS or BNST inhibits intake (Sweeney and Yang, 2015).

Our finding that postmeal inactivation of vHC neurons increases sucrose intake and meal frequency are consistent with previous findings showing that permanent vHC lesions, chemogenetic inactivation of the vHC dentate gyrus-CA3 region, and vHC infusions of a GLP-1 antagonist or ghrelin increase intake of standard chow (Davidson et al., 2009; Kanoski et al., 2013; Hsu et al., 2015a; Sweeney and Yang, 2015). These previous studies, however, did not restrict the manipulations to the postprandial period when the memory of a meal would be undergoing consolidation. As a result, the current study extends these previous findings by demonstrating that vHC neurons also influence intake of a palatable sucrose meal and that vHC neural activity inhibits energy intake during the postprandial period. Given that central infusions of muscimol inhibit neural activity for several hours (Martin, 1991; Arikan et al., 2002), it is likely that vHC neurons were inactivated throughout the experimental period. Thus, the present finding that muscimol increased meal size and intake throughout the experiment also suggests that vHC neurons inhibit intake once consumption is initiated. A 1 µL infusion volume was used in the present experiment to ensure that critical vHC subfields were not missed. This volume of muscimol decreases neural activity for ~1 mm, even though it has a larger diffusion radius (~1.5 mm, Martin, 1991; Martin and Ghez, 1999). Consequently, the present results cannot identify whether particular regions within vHC are critical. Although our results indicate that vHC neurons influence intake during the postprandial period, it is likely that these neurons also influence intake during the period prior to meal initiation. This possibility is supported by findings showing that the hormone ghrelin, which is released prior to intake and is considered an anticipatory signal for meal initiation (Hsu et al., 2016), increases intake when infused into vHC

(Kanoski et al., 2013). Moreover, blockade of vHC ghrelin receptors decreases intake of an anticipated meal (Hsu et al., 2015b).

Although there are several differences between the procedures used in the present vHC inactivation experiment and our previously published findings with dHC inactivation (Henderson et al., 2013) that preclude direct comparison, our combined findings suggest that vHC inactivation may produce bigger increases in intake than dHC inactivation. The reasons for these differences are unclear, but collectively the findings indicate that both dHC and vHC neurons suppress energy intake during the postprandial period. One question that arises is whether they do so through similar or different underlying mechanisms. Our finding that sucrose ingestion increases *Arc* expression in both dHC (Henderson et al., 2016) and vHC (present findings) neurons is consistent with the possibility that both dHC and vHC neurons regulate intake through a process that involves memory. It is possible that dHC neurons encode the “what, where, and when” components of a recently eaten meal (Eichenbaum, 2004; Shapiro et al., 2006); whereas, vHC neurons encode the affective/emotional aspects (Fanselow and Dong, 2010; Strange et al., 2014). Compared with our published findings in dHC that were based on tissue from the same animals, the current results suggest that sucrose ingestion produces less *Arc* expression in vHC than dHC. This is consistent with other findings that have compared *Arc* expression in dHC and vHC (Gusev et al., 2005; Nalloor et al., 2012, 2014) and with results suggesting that dHC expresses higher levels of synaptic plasticity than vHC (Papatheodoropoulos and Kostopoulos, 2000a, 2000bb; Maruki et al., 2001; Maggio and Segal, 2007).

Inactivation of dHC and vHC neurons could also increase intake through additional mechanisms that are not necessarily mutually exclusive. For instance, inactivation of dHC neurons could also influence meal timing by interfering with the ability to track the amount of time that has elapsed since the last meal (Itskov et al., 2011; MacDonald et al., 2011; Tam and Bonardi, 2012a, 2012bb; Tam et al., 2015). dHC and vHC inactivation could also interfere with satiety signaling. In rodents, large lesions that encompass dHC and vHC (Davidson and Jarrard, 1993; Davidson et al., 2010) as well as lesions restricted to either dHC or vHC (Hock and Bunsey, 1998) impair the ability to detect interoceptive cues. Similarly, patients with episodic memory deficits have a diminished ability to interpret and report internal energy state cues (Hebben et al., 1985; Rozin et al., 1998; Higgs et al., 2008). Evidence from intact humans provides indirect support for the involvement of memory dysfunction, however, by showing that HC-dependent memory does indeed influence future intake (Robinson et al., 2013a). Moreover, satiety cues are likely needed to form a memory of a meal, which is supported by findings showing that postprandial signals such as leptin increase synaptic plasticity in HC (Shanley et al., 2001; Harvey et al., 2006), and by our findings showing that sucrose intake increases dHC (Henderson et al., 2016) and vHC *Arc* expression (present findings). Additional data are needed to resolve whether dHC and vHC inactivation-induced increases in intake are due to impaired ability to detect interoceptive cues, a deficit in the interpretation or use of those cues, and/or impaired consolidation of the memory of the meal. It is also possible that vHC inactivation interfered with the retrieval of the non-rewarding postingestive outcomes of energy intake that occur toward the end of a meal (Davidson et al., 2014). This

possibility could be tested in the future, for instance, by determining whether vHC inactivation influences the intake of saccharin, which provides a similar sweet orosensation with minimal postingestive consequences (Byard and Goldberg, 1973; Mook et al., 1980; Renwick, 1985, 1986; Sclafani and Nissenbaum, 1985). Finally, it is also possible that vHC inactivation could have increased the motivation to eat in a manner that did not involve cognition or memory. For instance, complete hippocampal lesions decrease reward thresholds for ventral tegmental self-stimulation and increase the breakpoint for responding in a progressive ratio operant task (Schmelzeis and Mittleman, 1996; Kelley and Mittleman, 1999), suggesting that these lesions increase motivational and reward processes. Whether lesions restricted to vHC increase such processes is apparently unknown.

The finding that vHC inactivation increases energy intake raises the possibility that vHC dysfunction contributes to the development and maintenance of diet-induced obesity. Of note, increased meal frequency and snacking are positively correlated with increased body mass and obesity (Nielsen et al., 2002; Cutler et al., 2003; Nicklas et al., 2003; Murakami and Livingstone, 2015). Obese individuals consume snacks more frequently than their non-obese counterparts (Berteus Forslund et al., 2005), and increased consumption of sugar-sweetened beverages is positively associated with weight gain and obesity (reviewed in Malik et al., 2006; Malik et al., 2013). Excess consumption of sugars and fat impairs hippocampal-dependent memory in rats (Ross et al., 2009; Kanoski and Davidson, 2011; Ross et al., 2012; Darling et al., 2013) and may contribute to a vicious cycle wherein excess consumption of sugars impairs hippocampal function, thereby increasing future intake (Kanoski and Davidson, 2011).

In summary, these findings indicate that ingestion is associated with synaptic plasticity in vHC neurons during the postprandial period and that vHC neural activity during the period following a meal inhibits intake. Collectively, these findings suggest that vHC-dependent memory influences energy intake. Given that promoting the memory of meal reduces future intake (Robinson et al., 2013a, 2013bb; Robinson et al., 2014), understanding how cognitive factors such as memory influence energy intake may contribute to the development of new treatment options for disorders that involve altered ingestive behavior.

2.6 Acknowledgements

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2.7 Figures

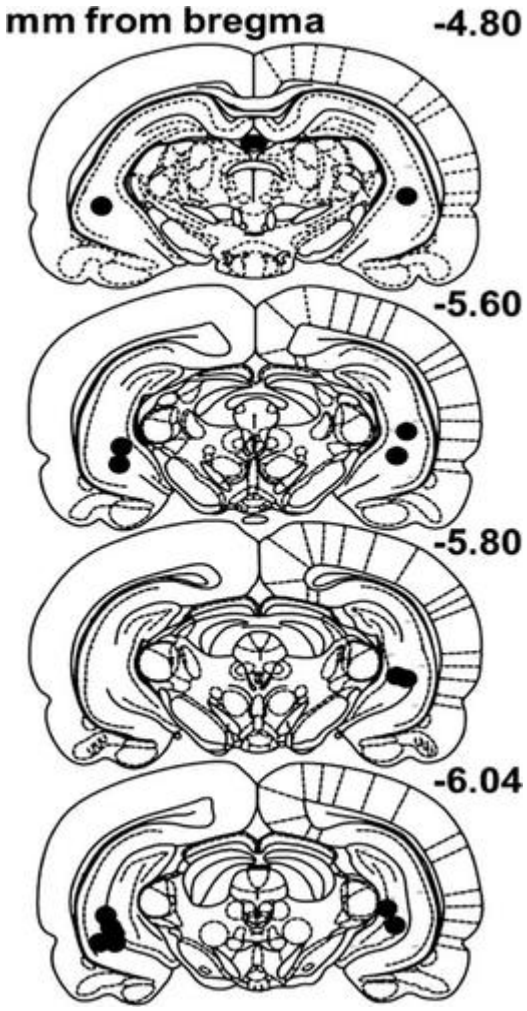

Figure 2.1: Estimated location of infusions in vHC.

Figures adapted from Paxinos and Watson, *The rat brain in stereotaxic coordinates (4th ed.), 1998, Academic Press/Elsevier, reproduced by permission.*

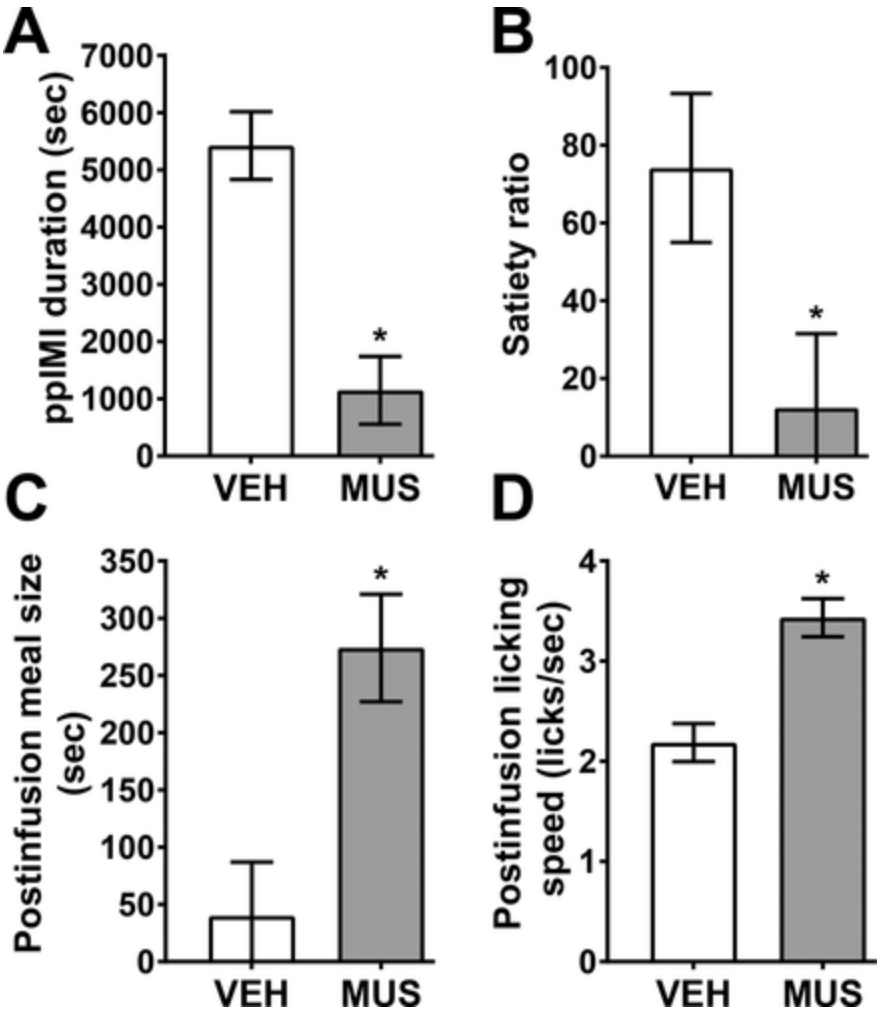

Figure 2.2: Postmeal vHC muscimol (MUS) infusions accelerate the initiation of the next sucrose meal and increase the amount of sucrose consumed during that postinfusion meal.

Compared to vehicle (VEH) infusions, vHC MUS infusions significantly (A) decreased the mean (+/−SEM) duration of the ppIMI and the (B) mean (+/−SEM) satiety ratio and increased C) the mean (+/−SEM) amount of sucrose consumed during the postinfusion meal and D) mean (+/−SEM) licking speed during that meal (*$P < 0.01$ vs. VEH).

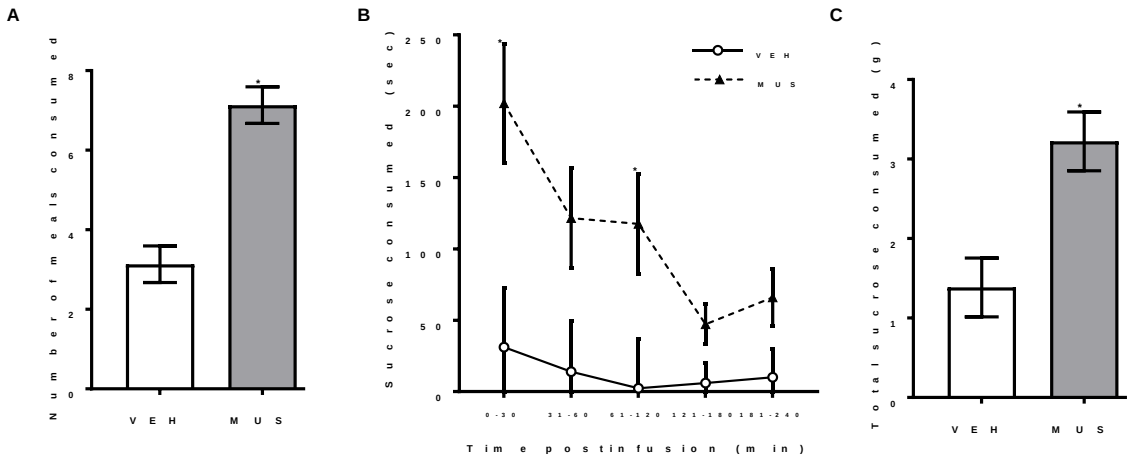

Figure 2.3: Postmeal vHC MUS infusions increase meal frequency and sucrose intake during the 4-hr test period.

Compared to VEH infusions, vHC MUS infusions significantly increased A) the mean (+/−SEM) number of sucrose meals, mean (+/−SEM) sucrose consumption during the first 2 hr of the experimental period, and C) the mean (+/−SEM) total amount of sucrose consumed (*$P < 0.01$ vs. VEH).

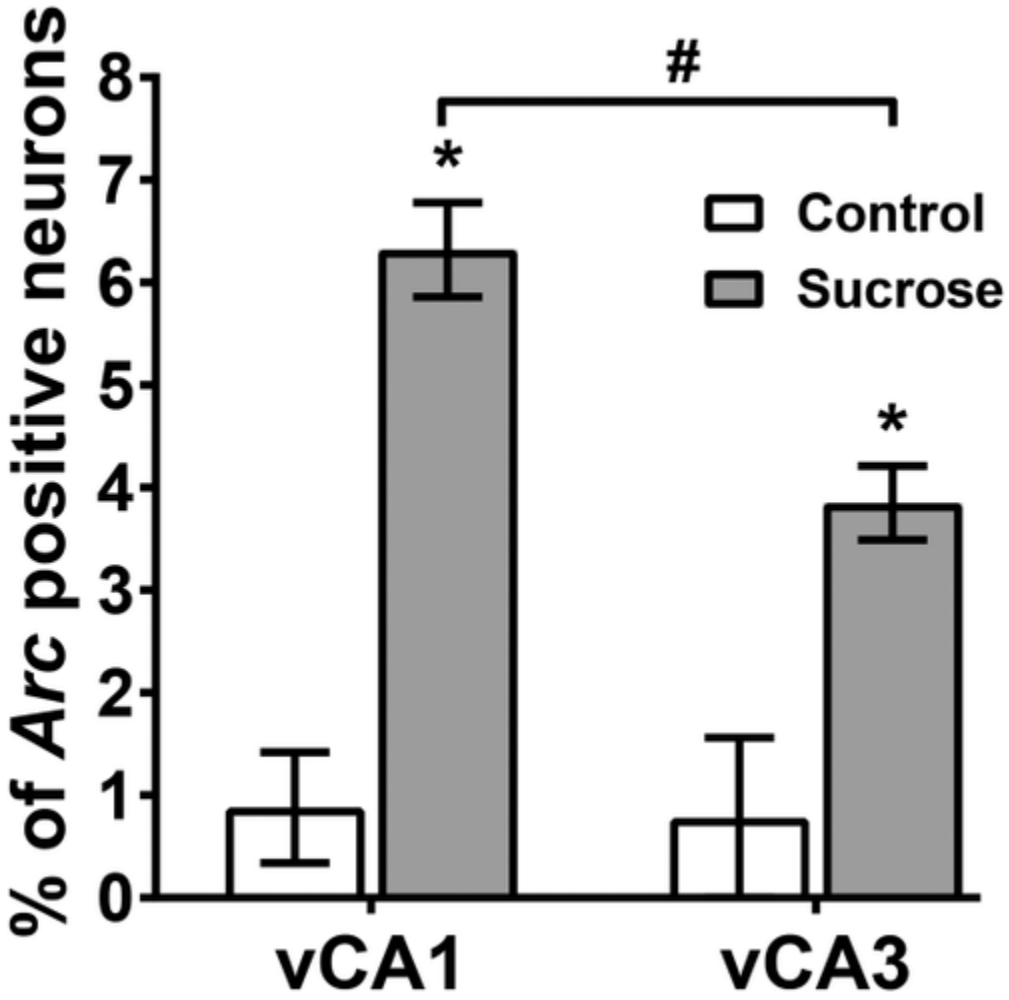

Figure 2.4: The mean (+/−SEM) percentage of Arc-expressing neurons in vCA1 and vCA3 is significantly higher in rats that just consumed a sucrose solution than in cage control rats.

Sucrose-associated Arc expression is higher in vCA1 than in vCA3 (*$P < 0.01$ vs. Control; # $P < 0.01$ vs. vCA1).

3 VENTRAL HIPPOCAMPAL NMDARS AND ARC INHIBIT FUTURE INTAKE IN RATS

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3.1 Abstract

There is a very limited understanding of how brain areas involved in cognition regulate energy intake. In humans impairing the encoding of a meal-related memory increases intake during the next meal. Previous work shows that ventral hippocampal (vHC) neurons, which are critical for affective and emotional memory, also inhibit energy intake during the postprandial period. It is unknown however if vHC regulation of energy intake employs the same molecular mechanisms necessary for memory formation and encoding. In the current study, we tested the hypothesis that vHC inhibition of future intake requires activation of glutamatergic N-methyl-D-aspartate receptors (NMDARs) and vHC *Arc* expression. Rats were given vHC infusions of either the NMDAR antagonist APV or Arc antisense oligonucleotides (ODNs), Compared to intake on days

when rats were given control infusions, rats given vHC APV or Arc ODNs consumed more during their first meal and did not accommodate for that increase by delaying the onset of their next meal (i.e., disrupted satiety ratio). These data show that vHC NMDARs and vHC *Arc* limit future intake in rats.

Key words: Sucrose, *Arc*, memory, NMDARs

3.2 Introduction

Growing evidence suggests that cognitive factors such as memory play a critical role in regulating energy intake. For instance, memory serves as record of recent intake and inhibits future intake. In humans, enhancing the memory of a meal reduces the amount of food consumed during the next feeding bout (Robinson et al. 2013). Conversely, consuming a meal while distracted impairs the encoding of the memory of that meal and increases subsequent feelings of hunger and the amount of food eaten during the next meal (Brunstrom and Mitchell 2006, Higgs and Woodward 2009, Oldham-Cooper et al. 2011). Patients with severe memory deficits do not remember having just eaten and will consume an additional meal when presented with food despite having just eaten to satiation (Hebben et al. 1985, Rozin et al. 1998, Higgs 2008).

The hippocampus (HC) is a brain region essential for memory processes (Fanselow and Dong 2010, Strange et al. 2014), and increasing evidence shows that the hippocampus is crucial for regulating energy intake (Henderson et al. 2013, Hannapel et al. 2017). The hippocampus can be anatomically and functionally divided into the dorsal hippocampus (dHC) and ventral hippocampus (vHC) (Moser and Moser 1998, Fanselow and Dong 2010, Strange et al. 2014). vHC neurons are critical for motivation, affect and emotional memory (Moser and Moser 1998, Bannerman et al. 2004, O'Mara et al. 2009, Barkus et al. 2010, Fanselow and Dong 2010, Kesner 2013).

One of the principal mechanisms for the long-term storage of information in the HC is the modification of synaptic efficacy (i.e., synaptic plasticity). Activation of glutamatergic N-methyl-D-aspartate receptors (NMDARs) is required for most forms of

HC synaptic plasticity (Malenka and Nicoll 1993). Activation of NMDARs increases intracellular Ca^{++} levels and initiates a series of downstream molecular cascades resulting in the transcription of new mRNA and the synthesis of several protein products aimed at increasing AMPA receptor function and elevating synaptic strength (Davis et al. 1992, Cammarota et al. 2000, Bast et al. 2005, Bevilaqua et al. 2005, Bloomer et al. 2008, Bourne et al. 2013). Pharmacological blockade of HC NMDARs inhibits synaptic plasticity and disrupts memory formation (Czerniawski et al. 2011, Czerniawski et al. 2012, Park et al. 2014). Moreover, feeding related hormones such insulin and leptin enhance NMDAR functionality (Liu et al. 1995, Shanley et al. 2001).

It is unclear, however, whether vHC neurons inhbit energy intake through a process that involves memory. vHC neurons are poised to integrate energy-related signals with mnemonic processes. vHC neurons contain receptors for numerous food-related signals, (Kanoski and Grill 2015) and project to several brain regions critical for food intake (Namura et al. 1994, Cenquizca and Swanson 2006, Radley and Sawchenko 2011, Hsu et al. 2015). vHC lesions increase food consumption and body mass (Davidson et al. 2009), and activation of vHC receptors for gut hormones affects food intake and food-related memory (Kanoski et al. 2011, Kanoski et al. 2013, Hsu et al. 2015, Hsu et al. 2017). Additionally, optogenetic excitation of vHC glutamatergic projections to the bed nucleus of the stria terminalis, lateral septum, or prefrontal cortex inhibit energy intake (Sweeney and Yang 2015, Hsu et al. 2017).

Findings from our laboratory indicate that vHC neurons inhibit energy intake during the postprandial intermeal interval (ppIMI; i.e., the time between two meals) (Hannapel et al. 2017). Specifically, inhibiting vHC neurons during the period shortly

after intake when the memory of a meal would be consolidated into a stable memory trace decreases the ppIMI duration and increases the amount consumed during the next meal (Hannapel et al. 2017). Sucrose consumption also increases *Arc* expression in vHC neurons (Hannapel et al. 2017). *Arc* is necessary for long-term vHC memory formation (Czerniawski et al. 2011, Czerniawski et al. 2012, Chia and Otto 2013) and is required for the consolidation of memory, but not for the initial learning of a behavior (Plath et al. 2006). Disrupting vHC *Arc* expression with antisense oligonucleotides (ODNs) impairs consolidation of memory across numerous HC-dependent behaviors (reviewed in (Bramham et al. 2010, Shepherd and Bear 2011). vHC *Arc,* therefore, may be critical for regulating intake during the ppIMI when a meal-related memory should be undergoing consolidation. If vHC neurons form a meal-related memory that inhibits future intake then pharmacologically blocking vHC NMDARs or disrupting vHC *Arc* should increase intake and decrease the time between meals.

3.3 Materials and Methods

3.3.1 *Experiment 1 – Does pharmacologically blocking NMDARs increase intake and decrease the interval between meals?*

3.3.1.1 Subjects

Adult male Sprague-Dawley rats (n = 15 postnatal day 52-58 upon arrival; Charles River Laboratories) were single-housed in Optirat® cages (Animal Care Systems). Unless otherwise stated, the rats were kept on a 12:12 hr light/dark cycle and given *ad libitum* access to pelleted food and water in their home cages. All procedures were performed in compliance with the NIH guidelines for care of laboratory animals

and approved by the Georgia State University Institutional Animal Care and Use Committee.

3.3.1.2 Stereotaxic Surgery

At least 1 week after arrival, the rats were anesthetized with 5% isoflurane (Henry Schein Impromed) in 1000 mL/min of oxygen (Airgas) and given penicillin (1500 IU, im; Henry Schein Impromed) and carprofen (5 mg/kg, sc; Henry Schein Impromed). Anesthesia was maintained with 1-3% isoflurane gas mixed in 500 mL/min oxygen for the duration of the surgery. Unilateral guide cannulae (8.5 mm long, 26-gauge; Plastics One, Roanoke, VA) were implanted aimed at the left or right vHC in a counterbalanced manner (AP: -5.3 mm, ML: ±5.1mm, DV: -6.4 mm from skull (Paxinos and Watson 2007). Guide cannulae were held in place by jewelers' screws (Plastics One, Roanoke, VA) and cranioplastic cement (DuraLay, Reliance Dental Mfg. Co.) and an obdurator (Plastics One, Roanoke, VA) was inserted into the cannula. The rats were given sterile saline (0.9%; 3.00 cc, sc; Hospira) at the end of surgery and allowed to recover for at least 1 week before behavioral training.

3.3.1.3 Sucrose exposure

To determine whether vHC NMDARs and *Arc* limit intake during the postprandial period, rats were given exposure to a 32% (w/v) sucrose solution at a specific time and place. Sucrose was used as the meal because 1) it is very palatable/rewarding to rats (Hajnal et al. 2004, Smith 2004), 2) its stimulus qualities are more specific than meals that include fats and proteins, 3) many of its peripheral and central processing sites and mechanisms have been identified (Levine et al. 2003, Smith 2004), 4) it cannot be

hoarded, and 5) vHC neurons control sucrose intake during the ppIMI and sucrose consumption increases vHC *Arc* expression (Hannapel et al. 2017).

On the first exposure day, the rats (n = 15) were brought to the testing room at the beginning of the light cycle, placed into polycarbonate testing cages (22 cm x 43 cm x 22 cm) that did not contain any chow but did have water, and were then given the sucrose solution 8 hr later for 10 min. Rats were exposed to sucrose in a similar manner on subsequent days with the exception that sucrose was presented 3 hr after the rats were placed into the testing cages instead of 8 hr. We started with an 8-hr period without chow in order to increase the likelihood that the rats would approach the bottle but then decreased it to 3 hr to be within the range of an average postprandial intermeal interval; (ppIMI; Snowdon 1969). The rats were exposed to the sucrose solution daily until they consumed the sucrose in less than 30 sec from initial presentation of the sucrose bottle for 3 consecutive days.

3.3.1.4 Testing days and infusions

Testing days occurred 24 hr after the last sucrose exposure day. The rats were placed in their experimental cages in the behavior room without food for 2.75 hr and then removed from the cage and given an intra-vHC infusion of vehicle (0.5 µl; phosphate-buffered saline, [PBS]; 0.25 µL/min, Cellgro, Manassas, VA) or D-APV (30 mM; Tocris). The injection needle extended 1.0 mm beyond the bottom of the guide cannula and was left in place for 2 min following the injection to facilitate diffusion. The rats were then returned to the experimental cage and then 15 min later they were given sucrose for 4 hr during which meal size and the interval between meals were recorded. This dose of D-APV impairs NMDAR-dependent memory when infused into vHC prior to

training in a memory task (Bast et al. 2005, McHugh et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Inglis et al. 2013). We reasoned that if dHC neurons are forming a NMDAR-dependent meal-related memory when they consume a meal, then it is necessary to inhibit dHC NMDARs prior to food consumption. A within-subject design was used wherein rats were given infusions of vehicle or D-APV in a counterbalanced order with 72 hr between infusions.

All intake data were recorded using a modified lickometer system that measured the change in system resistance when a rat licked from a sipper tube (Model 86062, Lafayette Instruments). The Activity Wheel Monitoring Program (Lafayette Instruments) recorded all sipper tube contacts. A sipper tube contact was operationally defined as any direct oral contact with the sipper tube longer than 3 sec (Thaw et al. 1998). This criterion improved scoring reliability by virtually eliminating all sniffs as contacts. A meal was defined as any bout containing at least 30 licks (Smith 2000, Hannapel et al. 2015). All sipper tube contacts were assumed to result in ingestion and the amount consumed was estimated indirectly by summing the duration of all sipper tube contacts during the meal. A meal was operationally defined as 5 consecutive minutes without any sipper tube contact. This criterion was used because after 5 min without consumption there is a very low probability that a rat will initiate eating again (Zorrilla et al. 2005, Fekete et al. 2007) and an increased probability of grooming, sniffing, rearing and resting behavior known as the behavioral satiety sequence (Antin et al. 1975, Thaw et al. 1998, Zorrilla et al. 2005).

Meal size was estimated by measuring the total amount of time spent in contact with the sipper tube during the meal, which excluded time spent not licking. Rats that

did not consume more than one meal were given a maximum ppIMI of 14400 sec (4 hr). To control for the effects of variations in the size of the preinfusion meal on the duration of the subsequent ppIMI, the satiety ratio was also calculated to estimate the ppIMI duration. The satiety ratio is an index of the amount of time spent not eating that is produced by the previous meal (Panksepp 1973, Zorrilla et al. 2005). It was calculated in the present experiment by dividing the ppIMI by the size of the preinfusion meal in seconds. Total sucrose intake was estimated by weighing the bottle before and at the end of the experimental session.

3.3.1.5 Histology

After the completion of the behavioral tests, the rats were deeply anesthetized using 5% isoflurane gas (Henry Schein Impromed, Oshkosh, WI) in 1000 mL/min of oxygen (Airgas, Inc., Atlanta, GA), decapitated and their brains removed and stored in formalin for at least 48 hr. Brains were then sectioned (50 µm) and two observers that were blind to the behavioral results examined the sections to estimate the infusion location 1.0 mm below the bottom of the cannula tract. Five rats in total were removed from Experiment 1 as a result of misplaced cannulae (Figure 1A, 1B).

3.3.2 *Experiment 2 – Does down-regulating vHC Arc increase meal size and decrease the interval between meals?*

To determine whether vHC *Arc* is necessary for vHC inhibition of energy intake, rats (n = 16) underwent the same surgical procedures as described in Experiment 1, with the exception that rats were implanted with bilateral guide cannulae. After recovery, the rats were exposed to a 32 % sucrose solution in a similar manner as Experiment 1, with the exception that rats were given less sucrose exposure (3 days) because

repeated sucrose consumption decreases expression of *Arc* mRNA in HC neurons (Henderson et al. 2016).

3.3.2.1 Testing days and infusions

On the 4th, day, rats were given bilateral infusions (0.5uL; 0.25 µl/min) of either an *Arc* antisense ODN (2 nmol/µl) or scrambled ODN (Integrated DNA Technologies). The antisense ODN targeted *Arc* mRNA bases 209-228 and was chosen based on effective knockdown of *Arc* mRNA and protein (Guzowski et al. 2000, Ploski et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Chia and Otto 2013). The scrambled ODN was composed of the same base composition in a randomized order. The rats were then placed in the experimental cages for 3 hr and then given the sucrose solution. The *Arc* ODN or scrambled control were given 3 hr prior to sucrose exposure based on previous studies showing that the ODN is most effective 3 hr after infusion (Guzowski et al. 2000, Czerniawski et al. 2011, Chia and Otto 2013). The rats were given both the *Arc* antisense or scrambled control with 72 hr between injections in a counterbalanced manner.

3.3.2.2 Quantitative real-time PCR (qt-PCR)

Twenty-four hours after the last sucrose testing day, the rats were given the scrambled control in one hemisphere and the *Arc* antisense in the other and then given access to water or sucrose for 10 min 3 hr later. Hemispheres were counterbalanced between animals. Fifteen minutes later the rats were euthanized with a lethal dose a pentobarbital (120 mg/kg; Henry Schein Impromed) and perfused transcardially with 4% paraformaldehyde (Thermo Fisher Scientific). Brains were harvested and left overnight in paraformaldehyde (4 ^{o}C) and then transferred to a 30% sucrose and ethylene glycol

antifreeze solution for at least 48 hr. The brains were sectioned (50 µm) using a cryostat (CM3050 S; Leica Biosystems) in two series separate series. The first series was mounted on gelatin-subbed slides and stained with thionin to examine cannulae placement as in Experiment 1 (Figure 1A, 1B). Seven rats were removed as a result of misplaced cannulae. Bilateral vHC tissue punches (0.5 mm; Leica Biosystems) were taken from the second series and processed for qt-PCR.

RNA was isolated and purified using mirCURY RNA isolation kit (Exiqon). RNA concentrations were determined using a NanoDrop-2000 spectrophotometer (ND-2000, Thermo Fisher Scientific). Total RNA was reverse transcribed using Transcriptor First Stand cDNA synthesis Kit (Roche) and stored at -20 ^{o}C overnight. qtPCR was performed with commercially available primers for the housekeeping gene GAPDH (PPR06557B-200, Qiagen) and for Arc (PPR44661A-200, Qiagen) using a FastStart Essential DNA Green Master Mix (Roche). Samples were run in duplicate per gene in a LightCycler 96 Instrument (Roche). The samples were pre-incubated for 10 min at 95 ^{o}C and run through 55 cycles of 3-step amplification consisting of 95 ^{o}C for 10 sec, 60 ^{o}C for 10 sec, and then 72 ^{o}C for 10 sec. Relative quantification of *Arc* was determined using the Pfaffl method (Livak and Schmittgen 2001).

3.3.2.3 Statistical Analyses

All statistical analyses and graphs were generated using IBM SPSS Statistics for Windows (IBM Corporation) and GraphPad Prism 7 for Windows (GraphPad Software). The behavioral data were tested for normality using Shapiro-Wilk tests and homogeneity of variance using Bartlett's tests. For both Experiment 1 and 2, total sucrose consumption and the number of meals consumed were analyzed using paired

t-tests and the remaining measures were analyzed using non-parametric Wilcoxon signed-rank tests. qtPCR data were first compared to a hypothetical value of 1 (i.e., no change in *Arc* expression), then a one-way ANOVA was performed to examine groups differences with Bonferroni multiple comparisons *post hoc* tests.

3.4 Results

3.4.1 *Experiment 1 - Premeal inhibition of vHC NMDARs increases the size of the first sucrose meal and accelerates the onset of the next meal.*

The results showed that premeal inhibition of vHC NMDARs with vHC APV infusions increased the size of the first sucrose meal ($Z = -1.9876$, $p = 0.0466$; Figure 2A) and decreased the time between the first and second meals (i.e., the ppIMI duration, [$Z = -2.0896$, $p = 0.0366$]; Figure 2B) and the satiety ratio ($Z = -2.8031$, $p = 0.0051$; Figure 2C), but did not significantly increase the size of the second meal ($Z = -1.6818$, $p = 0.0930$; Figure 2D). Compared to vehicle infusions, vHC APV infusions also increased the total number of meals consumed during the 4-hr recording period ($t(9) = 4.243$, $p = 0.0022$; Figure 2E) and the total amount of sucrose ingested ($t(9) = 2.461$, $p = 0.0361$; Figure 2F).

3.4.2 *Experiment 2 - Down-regulating Arc mRNA increases the amount of sucrose consumed and disrupts the relationship between meal size and the timing of the next meal.*

Compared to vHC infusions of the scrambled control, the premeal vHC infusions of the *Arc* ODN increased the size of the first meal that was consumed after the infusion ($Z = -2.1917$, $p = 0.0234$; Figure 3A) and decreased the satiety ratio ($Z = -2.1917$ $p = 0.0273$; Figure 3C), but did not significantly affect the duration of the ppIMI ($Z = -0.1400$,

p = 0.9453; Figure 3B), the size of the second meal (Z = -0.5331, *p* = 0.6523; Figure 3D), the number of meals consumed during the recording period (t(8) = 1.368, *p* =0.2084; Figure 3E), nor the total amount of sucrose consumed (Z = -1.4809, *p* = 0.1641; Figure 3F).

The qtPCR data showed that sucrose consumption increased vHC *Arc* expression as expected and, more importantly, that the *Arc* ODN decreased vHC *Arc* expression (Figure 4). Specifically, vHC *Arc* expression in the hemisphere injected with the scrambled control was higher in rats given access to sucrose than in rats given access to water (t(3) = 5.286, *p* = 0.0132). In rats given access to water, vHC *Arc* expression was significantly lower in the hemisphere injected with the vHC ODN compared to the opposite hemisphere that was injected with the scrambled control (t(3) = 5.353, *p* = 0.0332). A similar effect was observed in rats that had consumed sucrose (t(2) = 14.11, *p* = 0.0050).

3.5 Discussion

The current study is the first to show that vHC NMDARs and *Arc* inhibit energy intake. Specifically, the results show that inhibiting either vHC NMDARs or vHC *Arc* increased the size of the first meal that was consumed after the infusion and decreased the satiety ratio. The decrease in satiety ratio suggests that vHC APV or *Arc* antisense decreased satiation (Zorrilla et al. 2005). Collectively, these findings support the hypothesis that vHC neurons regulate energy intake through molecular processes required for memory formation.

We found previously that postmeal vHC infusions of a $GABA_A$ agonist decreased the ppIMI duration and satiety ratio and increased the amount consumed during the

subsequent second meal, whereas the present findings found that vHC APV and *Arc* antisense infusions did not increase the size of the second meal. This difference is not likely due to effects of the drugs wearing off before the second meal as APV inhibits NMDAR function for at least 100 min (Morris 1989) and the *Arc* antisense inhibits *Arc* expression for hours (Guzowski 2002). The differences observed between the two studies may be due, in part to differences in the timing of the infusions. Rats in the current study were given infusions prior to the initial sucrose consumption to block NMDAR and *Arc* function before any memory consolidation could occur. The premeal infusions of APV or anti-*Arc* in the present study caused rats to ingest significantly larger first meals upon sucrose presentation compared to when they were given control infusions, which may cause other satiety factors such as gastric distention to overcome hippocampal regulation and limit intake at the second meal (Moran 2006, Cummings and Overduin 2007, Moran and Dailey 2011).

In contrast to evidence in humans, the present findings suggest HC neurons limit intake during the consumption of a meal in addition to limiting future intake. For example, in humans distraction during the consumption of meal does not affect the size of the meal currently being eaten, but enhances subsequent feelings of hunger and increases the amount consumed during the next meal (Brunstrom and Mitchell 2006, Oldham-Cooper et al. 2011). Conversely, improving the memory of a meal during that meal decreases the amount eaten at the next meal (Robinson et al. 2014). The current findings, however, show that blocking vHC NMDARs and down-regulating *Arc* during the initial meal increases the amount consumed during that meal rather than during the next meal that is consumed. It is possible that the consolidation of meal-related memory

begins while the rats are consuming their first meal and that the rats continue to eat as they have impaired memory of consuming sucrose in the first place. The current findings are similar to evidence accumulated about patient H.M. and others with episodic memory deficits that would not remember eating after ingesting to satiation and would consume additional food if presented with food essentially extending the size of the meal at that sitting (Hebben et al. 1985, Rozin et al. 1998, Higgs 2008).

In summary, these findings indicate that mechanisms essential for vHC synaptic plasticity and memory formation also limit energy intake. These data also suggest that vHC neurons regulate energy intake during the consumption of a meal in addition to during the ppIMI. Understanding the mechanisms of how cognitive factors such as memory influence energy intake is critical for developing novel treatments for energy regulation disorders as worldwide obesity rates have more than doubled in the last 20 years (Ogden et al. 2014).

3.6 Acknowledgements

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3.7 Figures

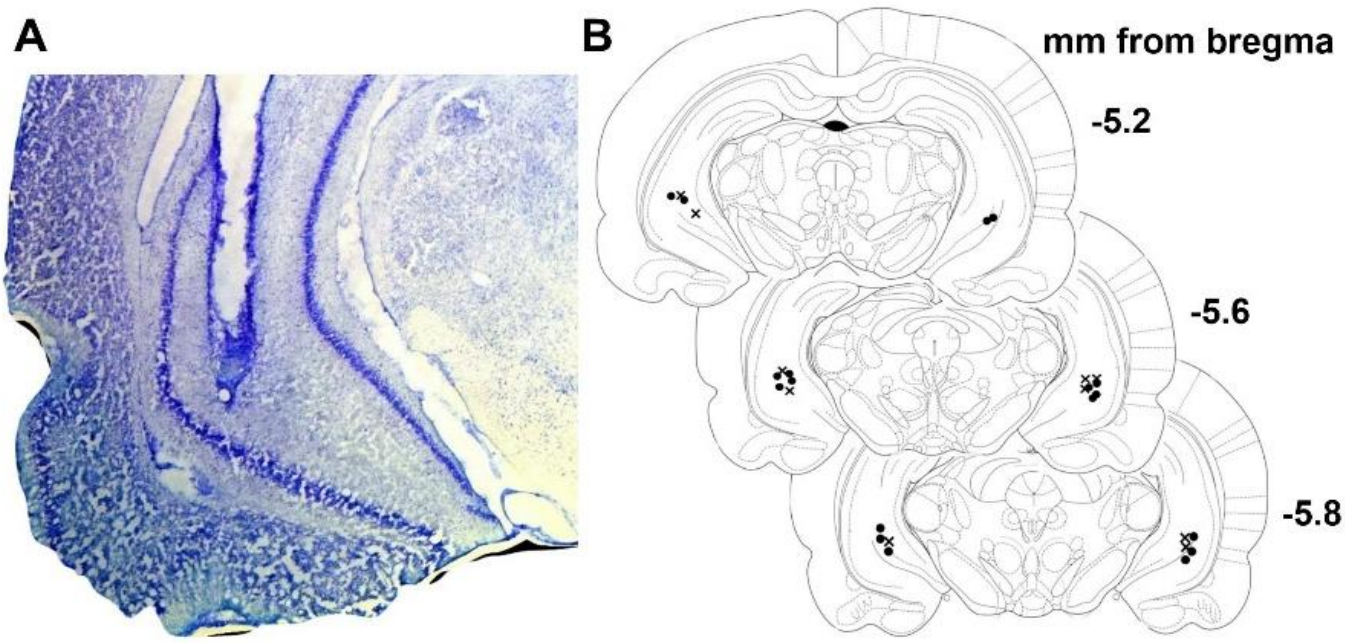

Figure 3.1: Cannula placement in vHC.

A, Representative depiction of vHC injection site in thionin-stained tissue. ***B,*** Schematic depiction of vHC unilateral cannulae placement relative to bregma (for APV injections (x) and bilateral cannulae (o) for *Arc* ODN injections).

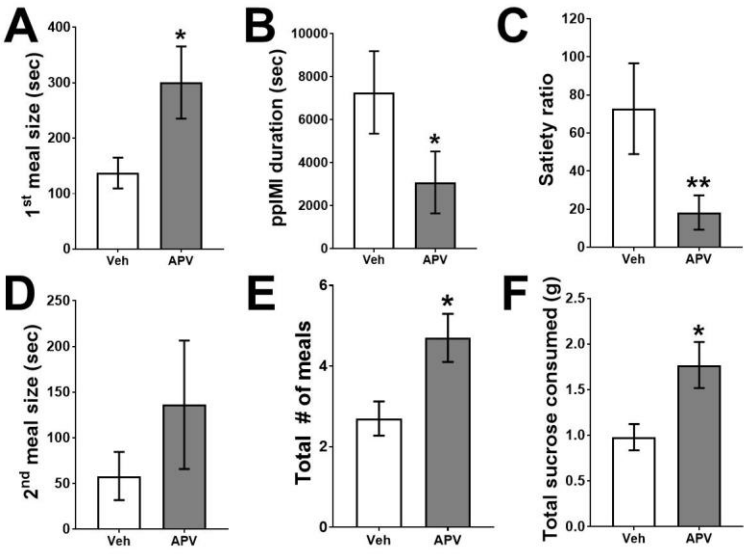

Figure 3.2: Premeal inhibition of vHC NMDARs increased the amount consumed during the first sucrose meal and accelerated the onset of the next meal.

A, Compared to vehicle infusions, premeal APV infusions (n = 10; within-subject) increased the size of the first meal that was consumed and ***B,*** decreased the ppIMI ***C,*** and satiety ratio. ***D,*** vHC APV infusions, however, did not affect the size of the second meal, ***E,*** but did increase the total number of meals ***F,*** and total amount of sucrose consumed during the 4-hr experimental period. *p<0.05; **p<0.01 vs Veh

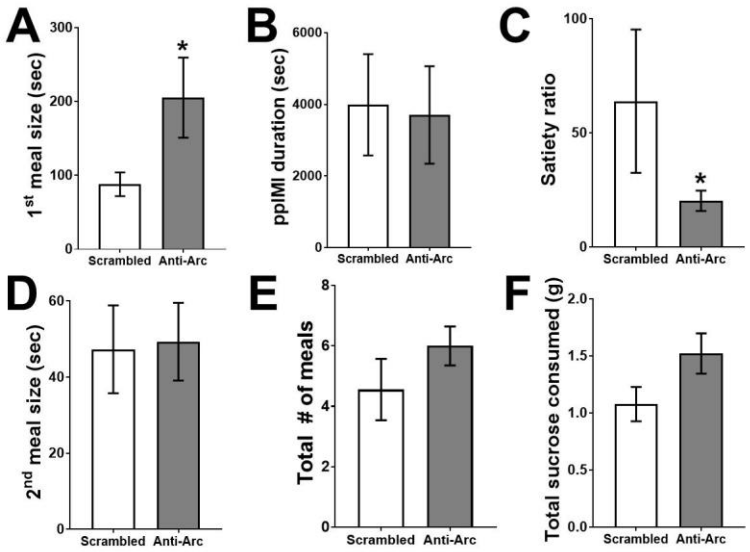

Figure 3.3: vHC Arc antisense infusions increased consumption during the first meal and disrupted the relationship between meal size and future meal timing. ***A,*** Compared to scrambled vHC infusions**,** vHC infusions (n = 9; within-subject) of an *Arc* antisense ODN increased the size of the first meal that was consumed, ***B,*** did not affect the ppIMI ***C,*** but did decrease the satiety ratio. ***D,*** Downregulating vHC *Arc* did not affect the size of the second meal, ***E,*** the total number of meals consumed ***F,*** nor the amount of sucrose consumed during the experimental period. *p<0.05 vs Scrambled control

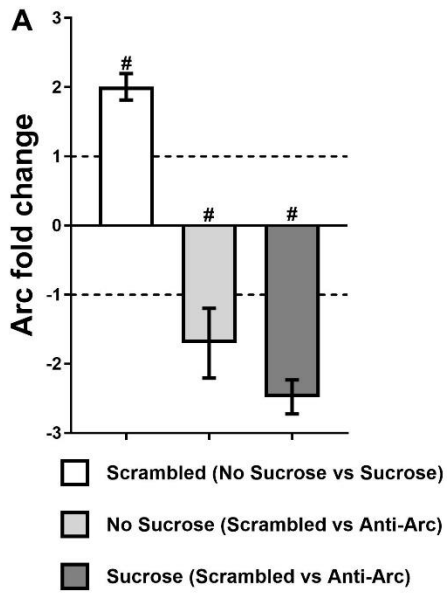

Figure 3.4: Sucrose consumption increases vHC Arc expression and vHC Arc antisense ODN infusions decrease vHC Arc expression.

A, Sucrose consumption increased vHC *Arc* expression in rats given the scrambled control (n = 4) compared cage-control rats given water (n = 4; No sucrose). In rats given access to water, vHC *Arc* expression was significantly lower in the hemisphere injected with *Arc* antisense (Anti-*Arc*) compared to the opposite hemisphere that was injected with the scrambled control (n = 4). A similar effects was observed in rats that had consumed sucrose. # $p<0.05$ vs hypothetical value of 1 (i.e., no change in vHC *Arc* expression).

4 POSTMEAL OPTOGENETIC INHIBITION OF DORSAL OR VENTRAL HIPPOCAMPAL PYRAMIDAL NEURONS INCREASES FUTURE INTAKE IN RATS

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4.1 Abstract

Memory of a recently eaten meal can serve as a powerful mechanism for controlling future eating behavior because it provides a record of intake that likely outlasts most physiological signals generated by the meal. In support, impairing the encoding of a meal in humans increases the amount ingested at the next eating episode. However, the brain regions that mediate the inhibitory effects of memory on

future intake are unknown. In the present study, we tested the hypothesis that dorsal (dHC) and ventral hippocampal (vHC) glutamatergic pyramidal neurons play a critical role in the inhibition of energy intake during the postprandial period by optogenetically inhibiting these neurons at specific times relative to a meal. Male Sprague-Dawley rats were given viral vectors containing CaMKIIα-eArchT3.0-eYFP or CaMKIIα-GFP and fiber optic probes into dHC of one hemisphere and vHC of the other. Compared to intake on a day in which illumination was not given, inhibition of dHC or vHC glutamatergic neurons after the end of a chow, sucrose, or saccharin meal accelerated the onset of the next meal and increased the amount consumed during that next meal when the neurons were no longer inhibited. Inhibition given during a meal did not affect the amount consumed during that meal or the next one but did hasten meal initiation. These data show that dHC and vHC glutamatergic neuronal activity during the postprandial period is critical for limiting future intake.

Key words: sucrose, saccharin, memory, postprandial

4.2 Significance Statement

Memory of a recently eaten meal provides a lasting record of recent intake and limits subsequent ingestion; however, the neural mechanisms underlying these mnemonic effects on future intake remain unknown. Here, we show that optogenetic inhibition of dorsal or ventral hippocampal pyramidal neurons induced after the end of a sucrose, chow or saccharin meal, when the memory of the meal would be undergoing consolidation, accelerated the initiation of the next meal and, importantly, increased the amount consumed during that next meal when the neurons were no longer inhibited. These findings show that neural activity in principal hippocampal neurons is necessary during the early postprandial period for limiting future intake.

4.3 Introduction

To date, the effort to understand the neural control of food intake has focused primarily on homeostatic and hedonic processes. In contrast, fewer studies have examined brain regions traditionally associated with other functions, such as memory. Yet, the memory of a recently eaten meal can serve as a powerful mechanism for controlling future eating behavior because it provides a record of recent intake that likely outlasts most physiological signals generated by the eating bout. Indeed, evidence from studies with humans suggests that impairing the episodic memory of a meal increases intake at the next eating episode and that enhancing meal-related memory has the opposite effect (Robinson et al. 2013). Moreover, patients with episodic memory-type amnesia do not remember eating and will eat an additional meal when presented with food despite just having eaten to satiety (Hebben et al. 1985, Rozin et al. 1998), and episodic memory deficits are associated with uncontrolled eating (Martin et al. 2017) and elevated body mass (Cheke et al. 2016).

The brain regions that mediate the inhibitory effects of ingestion-related memory on future intake are largely unknown. The principal cells of the hippocampus, pyramidal glutamatergic neurons, play a central role in memory (Izquierdo and Medina 1997, Zhu et al. 2014)**.** In particular, dorsal hippocampus (dHC) is critical for episodic memories of personal experiences (Fanselow and Dong 2010, Barbosa et al. 2012) and ventral HC (vHC) is important for emotional memory (Barkus et al. 2010, Fanselow and Dong 2010). As ingestion-related memories contain both mnemonic components, it seems likely that both regions contribute to the memory of a meal and limit future intake. Moreover, both dHC and vHC neurons are anatomically positioned to form a memory of

a meal because they express high concentrations of receptors for virtually every food-related signal (Kanoski and Grill 2015), receive neural impulses regarding energy status (e.g., taste and stomach distention; Xu et al. 2014), and project to most brain areas critical for energy regulation (Risold and Swanson 1996, Kishi et al. 2000, Cenquizca and Swanson 2006, Xu et al. 2014, Hsu et al. 2015).

In support of a role for these regions in regulating future intake, we reported previously that dHC or vHC infusions of the $GABA_A$ agonist muscimol given after the end of a first sucrose meal accelerated the onset of the second sucrose meal and increased the amount of sucrose consumed during that second meal (Henderson et al. 2013, Hannapel et al. 2017). The effects of muscimol are temporally imprecise (Martin 1991, Arikan et al. 2002) and the postmeal inactivation likely persisted throughout the postprandial period, during intake of the next meal, and beyond that. As a result, it is impossible to know whether these postmeal manipulations increased the amount consumed during the next meal by disrupting processes during the postprandial period or via an effect on intake during consumption of the second meal. Moreover, our prior work focused exclusively on scheduled sucrose meals presented during the light cycle. As a consequence, it is unclear whether the results extend to general homeostatic eating behavior in free-feeding animals during the dark cycle and whether the findings depend on the caloric value of the food. To address these issues and to investigate the specific role of principal hippocampal pyramidal neurons, the current study used an activity-guided optogenetic approach to inhibit dHC or vHC glutamatergic neurons in a temporally precise manner before, during, or after the consumption of a meal, which consisted of either sucrose, standard chow, or the non-caloric sweetener sacharin. This

allowed us to determine whether these neurons are critical for limiting future intake and more specificallty, to test whether neural inhibition restricted to the period following the consumption of a meal, when the memory of the meal would be undergoing consolidation, would increase subsequent intake at a later time when the neurons were no longer inhibited. Given that both dHC and vHC are implicated in memory consolidation (Oliveira et al. 2010, Holahan and Routtenberg 2011, Zhu et al. 2014), we predicted that postmeal inhibition of either dHC or vHC would hasten meal initiation and increase future intake.

4.4 Results

4.4.1 *Activation of eArchT3.0 in dHC or vHC inhibited neuronal firing in a temporally-specific, steady and reversible manner.*

We used patch-clamp electrophysiology in acute slice preparations to test the ability of eArchT3.0 to reversibly inhibit neuronal activity in dHC and vHC (Kheirbek et al. 2013). In the first approach, a depolarizing current injection was given for 4 sec and 556 nm light was applied to the cell during the middle 2 sec of the current injection. Cells from dHC (n = 6) and vHC (n = 5) were pooled and analyzed together because there were no differences between the effects of light on dHC vs. vHC (Cell location [i.e., dHC vs. vHC]: $F(1, 18) = 0.1680$, $p = 0.6920$; time: $F(2, 18) = 72.6440$, $p = 0.0010$, Cell location x time: $F(2, 18) = 0.0379$, $p = 0.9630$). Light application significantly decreased firing rate with a return to baseline activity following cessation of the light ($X^2(3) = 16.5450$, $p = 0.0009$, Figure 1A, B). In the second approach, brief depolarizing light pulses (50 msec) were applied to the cell at 1Hz and the cell was illuminated with constant 556 nm light for 10 min (i.e., the duration used in the behavioral experiments).

dHC (n = 2) and vHC (n = 2) cells were pooled for this analysis because there were no apparent differences between the effects of illumination of dHC vs. vHC cells. Continuous light significantly reduced the number of depolarizing steps that caused action potentials, with a return to baseline firing immediately upon termination of the light ($F(2, 6) = 137.0860$, $p = 0.0001$, Figure 1C, D).

4.4.2 *Histology*

A total of 25 rats were excluded from analysis in the sucrose (n = 6 ArchT3.0; n = 4 control virus), chow (n = 6 ArchT3.0; n = 4 control virus), and saccharin (n = 5 ArchT3.0) experiments due to incorrectly located ferrules or opsin expression, insufficient opsin expression or tissue damage in at least one hemisphere. Figure 2 provides a schematic depiction of the location of the ferrules in dHC and vHC of the same animal (Figure 2A), photomicrographs depicting representative virus expression in dHC (Figure 2B) and vHC (Figure 2C) and schematic depictions of virus distribution and ferrule locations for dHC (Figure 2D) and vHC (Figure 2E; Paxinos and Watson 2007).

4.4.3 *Optical inhibition of dHC or vHC glutamatergic neurons given DURING or AFTER the first sucrose meal decreased the latency to the second sucrose meal. Only inhibition given AFTER intake increased the amount consumed during the second meal when the neurons were no longer inhibited.*

The goal of this experiment was to identify when neural activity in dHC and vHC neurons is necessary for inhibiting intake. Illumination was provided to dHC or vHC during one of three epochs: 1) for 10 min before the rats were given sucrose (BEFORE first meal condition); 2) during the first 10 min of the first sucrose meal (DURING first meal condition); or 3) for 10 min after the end of the first sucrose meal (AFTER first

meal condition; Figure 3A). Intake was also assessed on another day in which the rats were attached to the laser, but not given illumination (NONE, non-illumination control condition). eArchT3.0 and control rats were given all seven treatment conditions (i.e. BEFORE, DURING, or AFTER the first meal in dHC or vHC and NONE) in a counterbalanced order with at least 48 hr separating each experimental day. This design allowed us to manipulate dHC and vHC in the same rat and decreased the number of animals needed by half. We found previously that unilateral dHC or vHC manipulations are sufficient to influence intake (Henderson et al. 2013, Hannapel et al. 2017). The latency to consume the first meal, the amount consumed during the first meal, the duration of the interval between the first and second meal (i.e., the postprandial intermeal interval [ppIMI]), the size of the second meal and licking rates (licks/sec) during each meal were measured. Compared to smaller meals, larger meals are followed by a longer ppIMI, which is referred to as the postprandial correlation (Le Magnen and Tallon 1963). Therefore, to control for the possible effects of differences between rats and between experimental days in the size of the first meal, we also used the satiety ratio (duration of ppIMI after first meal/size of the first meal) as a measure of the ppIMI.

Friedman tests indicated that optical activation of eArchT3.0 in dHC or vHC did not affect the latency to consume the first meal ($X^2(6) = 7.08$, $p = 0.3133$, the size of the first sucrose meal ($X^2(6) = 7.36$, p = 0.2887, Figure 3B) nor licking rates during the first meal ($X^2(6) = 7.07$, p = 0.3142), but did significantly affect the duration of the ppIMI ($X^2(6) = 39.4$, $p = 0.0001$, Figure 3C), magnitude of the satiety ratio ($X^2(6) = 26.4$, $p = 0.0002$, Figure 3D) and the size of the second sucrose meal ($X^2(6) = 35.3$, $p = 0.0001$,

Figure 3E). Dunn's *post hoc* tests indicated that the effects of optical inhibition on the ppIMI, satiety ratio, and size of the second meal depended on the timing and location of the inhibition. Specifically, compared to intake on a day in which illumination was not given (i.e., NONE), inhibition of vHC glutamatergic neurons given BEFORE the first sucrose meal accelerated the onset of the second sucrose meal (i.e., decreased the ppIMI: $p = 0.0001$ and satiety ratio: $p = 0.0003$, Figure 3C, D). In contrast, dHC inhibition given BEFORE the first sucrose meal did not affect these measures (ppIMI: $p = 0.4037$; satiety ratio: $p = 0.0770$). Inhibition of either dHC or vHC given DURING or AFTER intake of the first sucrose meal also decreased the ppIMI (DURING- dHC: $p = 0.0011$; vHC: $p = 0.0003$, AFTER- dHC: $p = 0.0001$; vHC: $p = 0.0001$, Figure 3C) and satiety ratio (DURING- dHC: $p = 0.0027$; vHC: $p = 0.0035$, AFTER- dHC: $p = 0.0020$; vHC: $p = 0.0001$, Figure 3D). Interestingly, only inhibition of dHC or vHC given AFTER the first sucrose meal increased the amount consumed during the second meal (dHC: $p = 0.0002$; vHC: $p = 0.0001$ Figure 3E). Inhibition given BEFORE or DURING the first sucrose meal did not affect the amount eaten during the next meal (BEFORE- dHC: $p = 0.9999$; vHC: $p = 0.1535$, DURING- dHC: $p = 0.5538$; vHC: $p = 0.9999$).

These effects of optical inhibition on the timing and amount of sucrose consumed were not due to the order of the manipulations across the experimental days (first meal size [$X^2(6) = 4.53$, $p = 0.6059$]; ppIMI [$X^2(6) = 9.00$, $p = 0.1736$]; satiety ratio [$X^2(6) = 9.81$ $p = 0.1327$]; second meal size [$X^2(6) = 3.68$, $p = 0.0576$]), and the increase in the size of the second meal was not due to increased licking rate during the consumption of that meal ($X^2(6) = 4.90$, $p = 0.0863$). Importantly, the Friedman tests showed that illumination of the control virus did not affect sucrose meal size or meal timing (first meal

size [$X^2(6) = 4.2$, $p = 0.6494$, Figure 3F]; ppIMI [$X^2(6) = 3.7$, $p = 0.7175$, Figure 3G]; satiety ratio [$X^2(6) = 5.16$ $p = 0.5233$, Figure 3H]; second meal size [$X^2(6) = 1.82$, $p = 0.9356$, Figure 3I]).

4.4.4 *Postmeal optical inhibition of dHC or vHC glutamatergic neurons also increased future intake of standard chow.*

This experiment determined whether dHC and vHC glutamatergic neurons also limit future homeostatic feeding behavior. We tested the effects of optical inhibition of dHC or vHC glutamatergic neurons given BEFORE, DURING, or AFTER the first chow meal of the dark cycle when rats typically eat their first major chow meal (Figure 4A; Clifton et al. 1984). As in the case of sucrose, the results of the Friedman tests indicated that optical illumination of eArchT3.0 did not affect the latency to consume the first chow meal ($X^2(6) = 3.05$, $p = 0.8021$) or the size of the first chow meal ($X^2(6) = 8.17$, $p = 0.2261$, Figure 4B), but did affect the timing of the next chow meal (i.e., the ppIMI duration [$X^2(6) = 39.90$, $p = 0.0001$, Figure 4C] and satiety ratio [$X^2(6) = 32.00$, $p = 0.0001$, Figure 4D]) and the amount consumed during the second meal ($X^2(6) = 23.90$, $p = 0.0005$, Figure 4E).

Dunn's *post hoc* tests indicated that the effects of inhibition were dependent on the timing of the inhibition relative to intake and on the anatomical location of the inhibition. dHC or vHC illumination given BEFORE intake of the first meal did not affect the timing of the second meal (ppIMI: dHC: $p = 0.9999$; vHC: $p = 0.9999$, Figure 4C, satiety ratio: dHC: $p = 0.9999$; vHC: $p = 0.9999$, Figure 4D) or the amount consumed during the second meal (dHC: $p = 0.9999$; vHC: $p = 0.1371$, Figure 4E). Illumination given DURING the first meal only affected the timing of the next meal but not the

amount consumed during that meal. Specifically, dHC inhibition given DURING the first chow meal significantly decreased the ppIMI (p = 0.0329, Figure 4C) and there was a trend for a similar effect of vHC inhibition given DURING the first meal (p = 0.0654, Figure 4C). vHC inhibition given DURING the first meal but not dHC inhibition given at that time decreased the satiety ratio (dHC: p = 0.7369; vHC: p = 0.0329, Figure 4D). Neither dHC nor vHC inhibition given DURING the first meal affected the size of the second meal (DURING- dHC: p = 0.9999; vHC: p = 0.7369, Figure 4E). Only illumination given AFTER the first meal promoted meal initiation and increased intake during that second meal. Optical inhibition of dHC or vHC given AFTER the first chow meal significantly also accelerated the onset of the next meal (i.e., decreased the ppIMI duration- dHC: p = 0.0001; vHC: p = 0.0001, Figure 4C and the satiety ratio- dHC: p = 0.0023; vHC: p = 0.0009, Figure 4D) and increased the amount consumed during the second meal (dHC: p = 0.0007; vHC: p = 0.0094, Figure 4E).

As in the case of sucrose, these effects of optical inhibition on the timing and amount of chow consumed were not due to the order of the manipulations across experimental days (first meal size [$X^2(6) = 3.45$, p = 0.9022]; ppIMI [$X^2(6) = 8.07$, p = 0.2329]; satiety ratio [$X^2(6) = 8.48$ p = 0.2052]; second meal size [$X^2(6) = 3.02$, p = 0.8058]). Of note, optical illumination of the control virus did not affect any of the intake measures (latency to the first meal [$X^2(6) = 5.17$, p = 0.5070]; first meal size [$X^2(6) = 5.17$, p = 0.5070, Figure 4F]; ppIMI [$X^2(6) = 2.19$, p = 0.9016, Figure 4G]; satiety ratio [$X^2(6) = 6.13$ p = 0.4089, Figure 4H]; second meal size [$X^2(6) = 2.57$, p = 0.8604, Figure 4I]).

4.4.5 *Postmeal optical inhibition of dHC or vHC glutamatergic neurons increased future intake of the non-caloric sweetener saccharin.*

In order to test whether postmeal optical inhibition increased sucrose and chow intake by impairing processing of postprandial interoceptive visceral cues (Davidson et al. 2014, Stevenson and Francis 2017), we tested the effects of postmeal dHC and vHC glutamatergic inhibition on intake of a 0.2% (w/v) saccharin solution. Saccharin is a non-caloric sweetener that has minimal postingestive consequences (Mook et al. 1980, Renwick 1985, Sclafani and Nissenbaum 1985, Renwick 1986). Importantly, in contrast to 32% sucrose and chow, whose intake is controlled by gastrointestinal and postabsorptive mechanisms (Strader and Woods 2005, Cummings and Overduin 2007), saccharin meal size and timing is controlled primarily by oral satiety (Mook et al. 1980, Kushner and Mook 1984, Sclafani and Nissenbaum 1985). The results indicated that activation of eArchT3.0 in dHC or vHC glutamatergic neurons given AFTER the first saccharin meal decreased the ppIMI ($F(1.01, 12.20) = 16.30$, $p = 0.0016$, Figure 5C) and satiety ratio ($F(1.03, 12.30) = 7.65$, $p = 0.0163$, Figure 5D) and increased the size of the second saccharin meal ($F(1.57, 20.40) = 5.05$, $p = 0.0226$, Figure 5E). Bonferroni *post hoc* analyses showed that this effect was produced by inhibition of either dHC or vHC (ppIMI- dHC: $p = 0.0026$; vHC: $p = 0.0040$, Figure 5C; satiety ratio- dHC: $p = 0.0270$; vHC: $p = 0.0270$, Figure 5D; second meal size- dHC: $p = 0.0035$; vHC: $p = 0.0492$, Figure 5E). The increases in second meal size were not due to an increase in licking rate ($F(1.46, 17.50) = 2.92$, $p = 0.2526$). As expected, inhibition given after the first meal did not affect the latency to first meal ($F(1.24, 14.90) = 1.46$, $p = 0.2526$) or the size of that meal ($F(1.76, 21.20) = 1.23$, $p = 0.3090$).

4.5 Discussion

The present results indicate that neural activity in principal dHC and vHC neurons is necessary during the early postprandial period for limiting future intake. Inhibition of dHC or vHC glutamatergic neurons given after the end of a meal increased the size of the subsequent meal when the neurons were no longer inhibited. The results also suggest that, although these neurons inhibit future intake, they do not limit current intake as optical inhibition given during a meal did not affect the amount consumed during that meal. Electrophysiological recordings showed that neural activity returned to baseline immediately upon termination of the 10 min of inhibition, supporting the inference that neural activity was not inhibited during intake of the second meal. Optogenetic inhibition increased future sucrose and chow consumption, indicating that dHC and vHC principal neurons inhibit future homeostatic and hedonic feeding behavior. The finding that postmeal optogenetic inhibition increased future saccharin intake suggests that the ability of dHC and vHC glutamatergic neurons to control future intake does not require postprandial visceral signals because saccharin ingestion produces minimal postingestive consequences and saccharin meal timing and size are determined primarily by oropharyngeal processes (Kushner and Mook 1984, Renwick 1985, Sclafani and Nissenbaum 1985, Renwick 1986). It would be interesting to test the effects of hippocampal inhibition on saccharin intake in rats given repeated exposure to saccharin because chronic saccharin intake can lead to subsequent overconsumption (Swithers 2015).

The present results also show that inhibition given during or after the consumption of a meal accelerated the onset of the next meal, suggesting that neural

activity at different time-points relative to ingestion influence meal initiation (i.e., during and after intake) versus future meal size (i.e., only after intake). The finding that inhibition given *during* a meal did not produce as robust an effect on future intake as did inhibition given *after* a meal also suggests that hippocampal neurons receive most of the neural signals necessary for controlling future intake during the postprandial period. The fact that postmeal inhibition did not commence until 5 min after the termination of the first meal suggests that hippocampal neural activity required to control future intake persists for more than 5 min after the meal. Moreover, the finding that inactivation given before intake of the first meal did not affect the amount eaten during that meal or the next one suggests that this neural activity does not endure until the onset of the next meal and that hippocampal neurons do not limit future intake through a process that involves retrieval of the memories of previously eaten meals and/or the processing of preprandial physiological signals.

Optical inhibition given for 10 min on multiple days did not appear to produce any long-term dysfunction. Our electrophysiological recordings showed that neural activity returned to baseline when the 10 min-illumination was terminated, consistent with the finding that 15 min of continuous eArchT3.0 illumination inhibits neural activity without producing desensitization or cellular damage (Huff et al. 2013, Tsunematsu et al. 2013). Our findings also showed that the effects of inhibition are specific to certain times and measures even though the rats were given inhibition at all time-points in a counterbalanced order, and we confirmed that intake did not change across experimental days. Importantly, our illumination protocol did not increase intake across all experiments in rats given the control vector.

Our previous results showing that dHC and vHC muscimol infusions given after a sucrose meal accelerated the onset of the next sucrose meal and increased sucrose intake (Henderson et al. 2013, Hannapel et al. 2017) did not distinguish between different periods relative to the meal and could have been due to the effects of the muscimol persisting through the next meal (Martin 1991, Arikan et al. 2002). Moreover, the findings did not specifically implicate principal neurons because muscimol could have inhibited several hippocampal neuronal types that express $GABA_A$ receptors (Semyanov et al. 2003, Glykys et al. 2007, Mann and Mody 2010). Thus, the use of temporally and genetically targeted manipulations in the present study significantly advances our understanding of hippocampal control of intake by providing compelling evidence that post-meal activity in principal hippocampal neurons is critical for limiting future intake.

Several lines of evidence suggest that postmeal hippocampal inhibition likely increased future intake by impairing the consolidation of the memory of the first meal, and that principal dHC and vHC neurons are a critical component of the neural mechanisms that underlie the ability of meal-related memory to inhibit future intake in human participants (Robinson et al. 2013). First, dHC or vHC manipulations given immediately after training in memory tasks impair subsequent retention (Oliveira et al. 2010, Holahan and Routtenberg 2011, Zhu et al. 2014), indicating that neural activity in dHC and vHC neurons during the period following an event is critical for memory consolidation. Second, dHC and vHC are necessary for many aspects of food/meal-related memories, such as food location, when food is available, and food reward (McDonald and White 1993, Kanoski and Grill 2015) . Third, our previous research

found that ingestion of sucrose or saccharin activates molecular processes critical for synaptic plasticity and memory formation in dHC and vHC during the postprandial period (Henderson et al. 2016, Hannapel et al. 2017, Ross et al. 2018). For instance, consistent with other types of learning (Czerniawski et al. 2011, Chia and Otto 2013), sucrose and saccharin ingestion increases hippocampal expression of activity-regulated cytoskeletal-associated protein (*Arc*) mRNA (Henderson et al. 2016, Hannapel et al. 2017), which is necessary for memory consolidation (Guzowski et al. 2000). Finally, the present finding that hippocampal inhibition given during a meal did not affect the amount consumed during that meal but did affect future intake is consistent with results from human participants showing that manipulating hippocampal-dependent memory encoding while eating has a bigger effect on intake at the next eating episode than on intake of the meal being remembered (Robinson et al. 2013).

Synaptic plasticity at hippocampal excitatory synapses is a critical mechanism underlying memory formation (Bailey et al. 2015). Increased synaptic strength in hippocampus augments functional connectivity between hippocampus and other brain regions (Canals et al. 2009), thereby providing a potential mechanism for hippocampal inhibition of intake. dHC neurons may modulate intake via longitudinal projections to vHC (Amaral and Witter 1989, Yang et al. 2014), which is the source of most hippocampal projections to brain areas involved in eating (Kanoski and Grill 2015). Indeed, activation of vHC glutamatergic projections inhibits feeding behavior (Sweeney and Yang 2015, Hsu et al. 2017). Of note, activation of dHC glutamatergic neurons increases neural activity in vHC, but not vice versa (Takata et al. 2015). This could account for similar effects of dHC and vHC inhibition and for finding that vHC inhibition

produced more effects than dHC inhibition. Given their roles in memory consolidation, it would be expected that inhibition of dHC or vHC given during the postprandial period would have a similar effect on future intake. This does not mean, however, that both hippocampal subregions serve identical roles in regulating feeding behavior. For instance, unlike dHC, vHC is also implicated in motivational aspects of feeding and is more sensitive to food-related hormonal signals than dHC (Kanoski et al. 2011, Kanoski et al. 2013, Fitzpatrick et al. 2016).

Compared to our knowledge of the neural controls of meal size, there is a large gap in our understanding of how the brain inhibits meal initiation and influences the duration of the ppIMI. The ppIMI determines meal frequency and thus also affects total intake, an important issue because increased meal frequency (i.e., snacking) coincides with the increased prevalence of diet-induced obesity (Cutler et al. 2003, Nicklas et al. 2003). The present results showed that hippocampal neural activity during and after ingestion is critical for influencing meal timing. vHC inhibition given before intake of a scheduled sucrose meal also accelerated the onset of the next meal but did not affect the timing of spontaneous chow meals, raising the possibility that neural activity in vHC but not dHC glutamatergic neurons during the anticipation of a highly palatable meal influences the future timing of these meals.

The current results also provide a mechanism by which hippocampal dysfunction and obesity produce a positive feedback loop that leads to more hippocampal pathology and weight gain. Excess intake of fats and/or sugars and obesity in rodents impair hippocampal synaptic plasticity (Grillo et al. 2011, Karimi et al. 2013) and hippocampal-dependent memory (Ross et al. 2009, Ross et al. 2012, Darling et al. 2013).

Hippocampal dysfunction, in turn, increases meal frequency and food intake (Davidson and Jarrard 1993, Clifton et al. 1998, Henderson et al. 2013, Hannapel et al. 2017) and promotes weight gain (Sample et al. 2016). In humans, being overweight or obese is associated with hippocampal atrophy (Cherbuin et al. 2015) and episodic memory deficits (Cheke et al. 2016), and enhancing the memory of a meal may be a promising strategy for limiting intake and promoting weight loss (Robinson et al. 2013, Robinson et al. 2014).

4.6 Materials and Methods

4.6.1 *Subjects*

Adult male Sprague-Dawley rats (N = 94; postnatal day 52-58 upon arrival; Charles River Laboratories) were single-housed in Optirat® cages (Animal Care Systems). Unless otherwise stated, the rats were kept on a 12:12 hr light/dark cycle and given *ad libitum* access to pelleted food and water in their home cages. All procedures were performed in compliance with the NIH guidelines for care of laboratory animals and approved by the Georgia State University Institutional Animal Care and Use Committee.

4.6.2 *Viral Vectors*

Recombinant serotype 5 adeno-associated virus (rAAV5) vectors containing CaMKIIα-eArchT3.0-eYFP or the control CaMKIIα-GFP (University of North Carolina Vector Core) were stored in aliquots (-80^{o}C) until surgery. In the hippocampus, the CaMKIIα promoter limits expression to glutamatergic pyramidal cells (Butler et al. 2016). Illumination of transduced neurons activates the hyperpolarizing outward proton pump

eArchT3.0, producing strong neural inhibition (Deisseroth 2011, Yizhar et al. 2011, Huff et al. 2013).

4.6.3 *Stereotaxic Surgery*

At least 1 week after arrival, the rats were anesthetized with 5% isoflurane (Henry Schein Impromed) in 1000 mL/min of oxygen (Airgas) and given penicillin (1500 IU, im; Henry Schein Impromed) and carprofen (5 mg/kg, sc; Henry Schein Impromed). Anesthesia was maintained with 1-3% isoflurane gas mixed in 500 mL/min oxygen for the duration of the surgery. A 33-gauge injection needle was used to deliver the rAAV5 (0.5 µl) into dHC (AP:-3.7 mm, ML:+2.8 mm: DV:-4.0 mm from skull surface; Paxinos and Watson 2007) of one hemisphere and vHC of the other (AP:-5.3 mm, ML:+5.1 mm, DV:-7.4 mm). These coordinates were selected based on previous research demonstrating that manipulations of these areas within the hippocampus impact memory processes (Bast et al. 2001, Oliveira et al. 2010, Czerniawski et al. 2011, Holahan and Routtenberg 2011, Chia and Otto 2013, Zhang et al. 2014). The hemispheres were counterbalanced across rats and the virus containing the same construct was injected in both hemispheres. The injection needle was left in place for 5 min after the end of the infusion to facilitate diffusion and the rats were given sterile saline (0.9%; 3.00 cc, sc; Hospira) at the end of surgery.

For rats used in the behavioral experiments, a second surgical procedure was performed at least 2 weeks later to implant fiber optic probes at each injection site. The probes were constructed using previously described procedures (Sparta et al. 2011, Huff et al. 2016). Briefly, a fiber optic (200 µm core; ThorLabs) was glued into a stainless-steel fiber ferrule assembly (Precision Fiber Products), and the ferrules were

affixed to the head using surgical screws and dental acrylic. Plastic dust caps (Precision Fiber Products) were placed on each ferrule to protect the fiber optic core. Rats were given at least 1 week of recovery prior to behavioral testing.

4.6.4 *Slice preparation and electrophysiology*

Patch-clamp electrophysiology recordings in acute dHC or vHC brain slice preparations were used to confirm the ability of eArchT3.0 to reliably and reversibly inhibit neuronal firing. Three to four weeks after the eArchT3.0 injections, rats (n = 5) were anesthetized with ketamine/xylazine (93/7 mg/kg, ip, Henry Schein) and transcardially perfused with ice-cold, carbogen (95% O2/5% CO2, AirGas)-saturated cutting solution. The brain was then removed and 300 µm coronal (dHC) or horizontal (vHC) brain sections were cut in carbogen-saturated ice cold cutting solution using a vibrating-blade microtome. After sectioning, the brain slices were transferred to carbogen-saturated aCSF and incubated at ~35 ^{o}C for 30 min. The sections were kept at room temperature until they were transferred to the perfusion chamber for recording. The sucrose cutting solution contained (in mM): sucrose 205, KCl 2.5, NaH2PO4 1.25, MgCl2 7.5, CaCl2 0.5, glucose 11.1, and NaHCO3 21.4. The aCSF contained (in mM): NaCl 126, KCl 2.5, CaCl2 2.4, NaH2PO4 1.2, MgCl2 1.2, glucose 11.1, NaHCO3 21.4. In a subset of experiments, kynurenic acid was included in the cutting solution (500-700 mM) and during the initial 35^{o}C incubation in aCSF (1 µM).

For electrophysiology recording, a brain slice was transferred to the recording chamber and perfused constantly with room temperature, carbogen-saturated aCSF at a flow rate of 1.5 - 2.0 ml/min. Recordings were made using a potassium gluconate-based internal solution containing (in mM): K Gluconate 128, HEPES 10.0, NaCl 10.0,

MgCl2 1.0, EGTA 0.1, (Mg) ATP 2.0, (Na) GTP 0.3, and creatine phosphate 10.0. Electrodes had a resistance of 3.4 – 4.3 MΩ when filled with the potassium gluconate internal solution. Series resistance values ranged from 5 – 15 MΩ, and experiments were terminated and the cell excluded from analysis if the series resistance exceeded 20 MΩ. The data were sampled at 10 kHz and filtered at 2.6 kHz using an Axon MultiClamp 700B amplifier and Axograph X software. Neurons expressing eArchT3.0 were identified by eYFP fluorescence and were patch-clamped under gradient contrast optics. A ferrule fiber optic probe was connected to the laser and positioned above the slice at a ~30-45-degree angle to activate eArchT3.0 during the electrophysiology recordings. After identification of a light-activated current in voltage clamp, neurons were recorded in the current-clamp configuration, and two approaches were used to test for the light-induced inhibition of neuronal firing (Kheirbek et al. 2013). 1) Neurons (n=11) were given a depolarizing current injection for 4 sec and green light (556 nm) was applied to the slice during the middle 2 sec. The firing rate during the depolarizing current injection was measured before, during, and after light application, and the rates were normalized to the firing rate before light activation to allow for comparison between cells. 2) Brief, depolarizing current injections (50 ms) were applied to the neurons (n=4) at 1 Hz to reliably elicit an action potential. After stable action potential generation was achieved for a minimum of 2.5 min, green light (556 nm) was continuously applied for 10 min (i.e., the duration used for the behavioral experiments), followed by additional monitoring for at least 2.5 min after the end of the light application. Action potential fidelity was calculated by determining the % of current injections eliciting an action potential at baseline, during light application, and in the post-light period. Cells were

only included in the analyses if the recording remained stable during the entire experiment. Cells that became unstable or died during the course of the recording were not included in the analyses.

4.6.5 *Optical inhibition during behavior*

Rats were connected to the laser by attaching their ferrules to a fiber optic leash using a Quick-Release interconnector (ADAF2; ThorLabs). The leash was attached to an optical commutator (RJPFF2; ThorLabs) allowing free rotation of the optic leashes. A FC/PC fiber coupler (Opto Engine LLC) connected the rotary joint to the laser source (200 mW DPSS laser, 556 nm; Opto Engine LLC). Light output was adjusted to allow for 10 mW from the fiber tip (Yizhar et al. 2011, Huff et al. 2013, Huff et al. 2016) and was measured using an optical power meter (PM20A; ThorLabs). Ten mW light output produces ~1 mW/mm^2 of light up to 1 mm from the fiber tip and illumination (556 nm) activates eArchT3.0 in at least 0.4 mm^3 of tissue (Yizhar et al. 2011).

4.6.6 *Sucrose consumption*

The effects of optical inhibition of dHC or vHC glutamatergic neurons on intake of 32% (w/v) sucrose solution were tested because 1) it is very palatable/rewarding to rats (Hajnal et al. 2004), 2) its stimulus qualities are more specific than meals that include fats and proteins, and 3) it cannot be hoarded. To rule out any effects of novelty and to ensure that rats reliably consumed sucrose upon presentation, the rats (eArchT3.0: n = 20; illumination-alone/no opsin control: n = 11) were exposed to the sucrose solution for 5 days prior to the optical manipulations. On the first exposure day, the rats were brought to the testing room at the beginning of the light cycle, placed into polycarbonate testing cages (22 cm x 43 cm x 22 cm) that did not contain any food, and then were

given sucrose 8 hr later for 10 min. The same procedure was repeated for the next 4 days, with the exception that sucrose was presented 3 hr after they were placed into the testing cages. We started with an 8-hr period without chow in order to increase the likelihood that the rats would approach the bottle, but then decreased it to 3 hr to be within the range of an average ppIMI (Snowdon 1969).

On the experimental days, the rats were placed in the testing cages without food and then given sucrose 3 hr later. They were connected to the laser 15 min before the sucrose was presented and were given sucrose for the duration of the 4-hr experimental period. The testing cages were equipped with a modified lickometer system that measured the change in system resistance when a rat licked from a sipper tube (Model 86062, Lafayette Instruments, Lafayette, IN). An experimenter measured latency to the first tube sipper tube contact using a Precision Solid State Time-It stopwatch (Petroleum Analyzer Company, L.P., Houston, TX). The Activity Wheel Monitoring Program (Lafayette Instruments) recorded all sipper tube contacts, which were operationally defined as any direct oral contact with the sipper tube longer than 3 sec (Thaw et al. 1998). This criterion improved scoring reliability by virtually eliminating all sniffs as contacts. A meal was defined as any bout containing at least 30 licks (Smith 2000, Hannapel et al. 2017). All sipper tube contacts were assumed to result in ingestion and the amount consumed was estimated indirectly by summing the duration of all sipper tube contacts during the meal. Rats that did not consume a second meal were given a ppIMI score of 4 hr minus the latency to consume their first meal and the duration of that meal.

In order to effectively time the AFTER first meal manipulations, it was critical to distinguish pauses within a meal from the end of a meal. Previous work indicates that when rats stop ingesting for 5 consecutive min there is a low likelihood that they will initiate eating again at that time (Zorrilla et al. 2005, Fekete et al. 2007) and a high probability that they will exhibit a progression of active grooming and resting behaviors known as the behavioral satiety sequence (Kushner and Mook 1984, Zorrilla et al. 2005, Fekete et al. 2007). Based on this evidence, a meal was operationally defined as any period of consumption of at least 30 licks followed by 5 min consecutive without licking (Smith 2000, Hannapel et al. 2017). One significant consequence of this operational definition is that 5 min had to elapse before the experimenter could know that the first meal was terminated and thus inhibition given AFTER the first meal condition was actually started 5 min after the end of the first meal. Also, in order to restrict the inactivation to the postprandial period, the laser was turned off before 10 min if rats began to consume their second meal during the illumination. For inhibition given DURING the first meal, the laser was turned off before 10 min if the rats stopped eating during illumination in order to restrict inhibition to ongoing intake.

4.6.7 *Chow consumption*

Rats (eArchT3.0: n = 18; control: n = 8) in this experiment were placed on a reverse light-cycle schedule (12:12 hr dark/light) upon arrival from the vendor and after 1 week of acclimation were given 3 days of habituation to the experimental procedures prior to behavioral testing. Specifically, the rats were moved to an illuminated testing room and placed in testing cages that did not contain chow 20 min before the end of the

light phase. After 20 min, the room lights were turned off, a red light was turned on, and a glass Petri dish containing standard chow was placed into the cage for 2 hr.

On experimental days, the rats were brought to the testing cages 20 min before the end of the light phase, connected to the laser, and then given chow 15 min later. Illumination was provided to dHC or vHC for 10 min either before chow was presented (BEFORE first meal condition), as soon as the rats started to ingest their first chow meal (DURING first meal condition), or after they stopped consuming their first chow meal (AFTER first meal condition). A meal was operationally defined as any period of consumption of at least 0.25 g of chow followed by 5 consecutive minutes without ingestion (Zorrilla et al. 2005, Kanoski et al. 2013, Hsu et al. 2015, Hsu et al. 2017). An experimenter blind to virus condition manually recorded the timing and amount of intake for 2 hr after chow presentation, which entailed weighing the dishes after each 5 min pause in eating. Rats that did not consume a second meal were given a ppIMI score of 2 hr minus the latency to consume their first meal and the duration of that meal.

4.6.8 *Saccharin Intake*

As in the sucrose experiment, rats (eArchT3.0: n = 13) were given 5 days of pre-exposure to the saccharin solution, and then on the experimental days they were given either no illumination or dHC or vHC illumination for 10 min after they consumed their first saccharin meal (AFTER first meal condition) in a counterbalanced order.

4.6.9 *Histology*

After the completion of the behavioral experiments, the rats were euthanized with a lethal dose a pentobarbital (120 mg/kg; Henry Schein Impromed) and perfused transcardially with 4% paraformaldehyde (Fisher). Brains were harvested and left

overnight in paraformaldehyde (4 ºC) and then transferred to a 30% sucrose and ethylene glycol antifreeze solution for at least 48 hr. The brains were sectioned (50 µm) using a cryostat (CM3050 S; Lieca Biosystems) and mounted on gelatin-subbed slides using a Mowiol and DABCO antifade medium (Sigma Aldrich). dHC and vHC images were obtained using a fluorescent microscope (Axio Zoom V16; Zeiss), and viral expression and ferrule placement were visualized using Zeiss AxioVision imaging software (Carl Zeiss).

4.6.10 *Statistical analyses*

Only rats that had successful placements in both dHC and vHC and that underwent all optical inhibition treatment conditions were included in the analyses. Intake in rats given illumination of CaMKIIα-eArchT3.0-eYFP or the control CaMKIIα-GFP BEFORE, DURING, OR AFTER the first meal was compared to intake on a non-illumination day (i.e., NONE; within-subject design for each construct). All statistical analyses and graphs were generated using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corporation), SigmaStat (v11.0, Systat Software, Inc.) and GraphPad Prism 7 for Windows (GraphPad Software). For the electrophysiology, the brief 2-sec inhibition data were analyzed using a repeated-measures ANOVA on ranks and the 10-min inhibition exposure data were analyzed using a repeated-measures ANOVA with a Tukey's post-hoc test. The behavioral data were tested for normality using and homogeneity of variance using Shapiro-Wilkes and Bartlett's tests, respectively. The results of these tests indicated that sucrose and chow intake measures required non-parametric analyses. Consequently, these data were analyzed with Friedman tests and Dunn's multiple comparisons *post hoc* tests were used to compare each optogenetic

condition to the non-illumination condition. These data are represented in the figures as box-and-whiskers plots with median values placed in the center of each box, with the whiskers representing the minimum and maximum values. The saccharin data were normally distributed and had homogeneous variance and were thus analyzed with one-way repeated-measures ANOVAs with Geisser-Greenhouse correction and Bonferroni's corrections for multiple comparisons. These data are also represented in the figures as box-and-whiskers plots to facilitate comparison with the sucrose and chow intake data.

4.7 Acknowledgments

This work was supported by National Science Foundation research grant IOS1121886 (MBP), National Institutes of Health research grants DK114700 (MBP) and MH104384 (RTL), a GSU Center for Obesity Reversal predoctoral fellowship (RCH) and the Center for Behavioral Neuroscience.

4.8 Figures

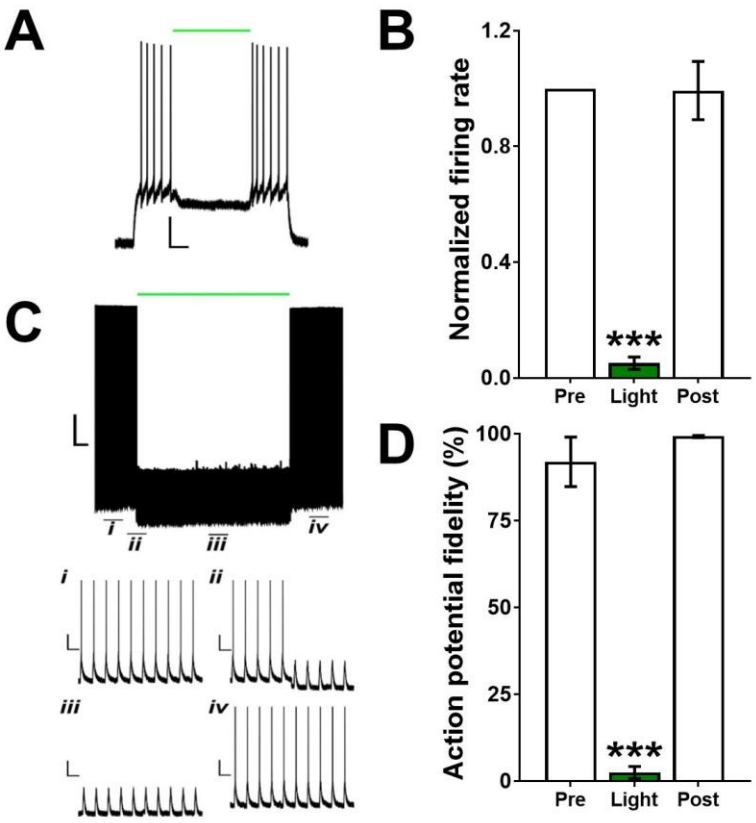

Figure 4.1: Optical stimulation of eArchT3.0 produced steady, temporally-specific, and reversible inhibition of dHC and vHC glutamatergic neurons.

A**,** Sample light-induced inhibition of a vHC-eArchT3.0-expressing neuron for 2 sec (green line). Scale bar 20 mV/0.5sec. ***B*****,** Light application for 2 sec significantly decreased the mean (±SEM) firing rate of dHC and vHC-eArchT3.0 expressing neurons during that 2-sec period (n = 11; dHC & vHC combined). ***C*****,** Sample light-induced inhibition of a vHC-eArchT3.0 expressing neuron for 10 min (green line); **C*i,*** at baseline, **C*ii,*** before, **C*iii,*** during and **C*iv,*** after light. Scale bars 20 mV/1 min (*i-iv*: 20 mV/1 sec). ***D*****,** Light application for 10 min significantly decreased mean (±SEM) action potential (AP) fidelity (n = 4; dHC & vHC combined). ****p*<0.0005 vs. Pre and Post.

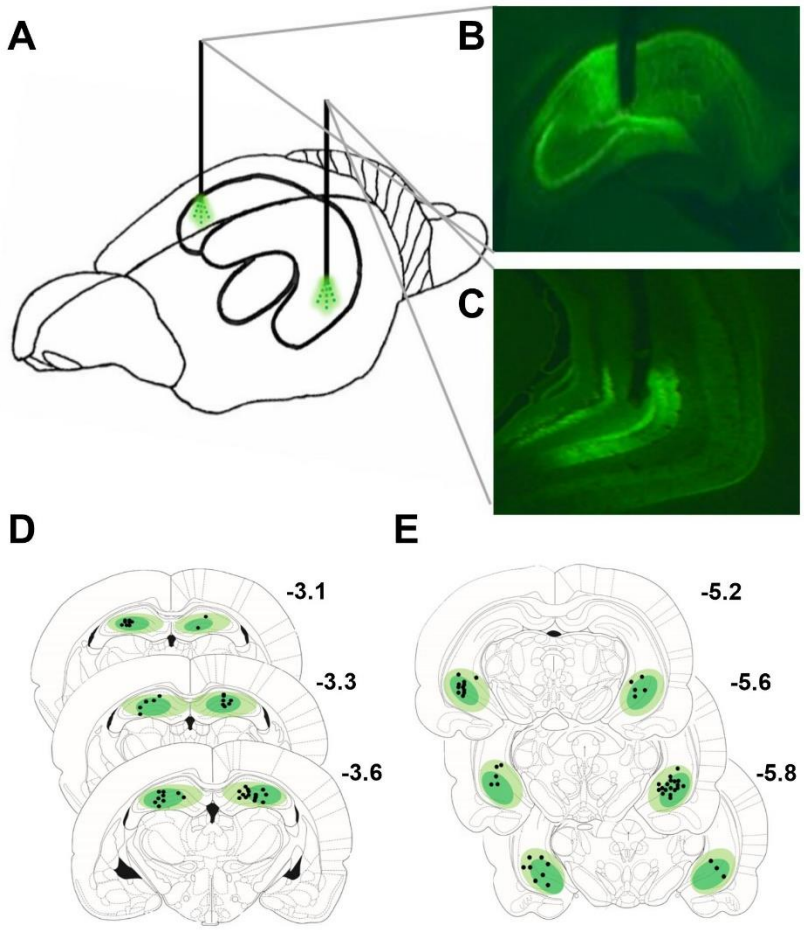

Figure 4.2: Schematic of ferrule placement and representative histological images.

A, Depiction of ferrule locations in dHC and vHC of the same rat. ***B,*** Representative image of robust eArchT3.0-eYFP expression and ferrule location in dHC and in ***C,*** vHC. ***D,*** Schematic depiction of virus expression and ferrule placement relative to bregma in dHC and in ***E,*** vHC.

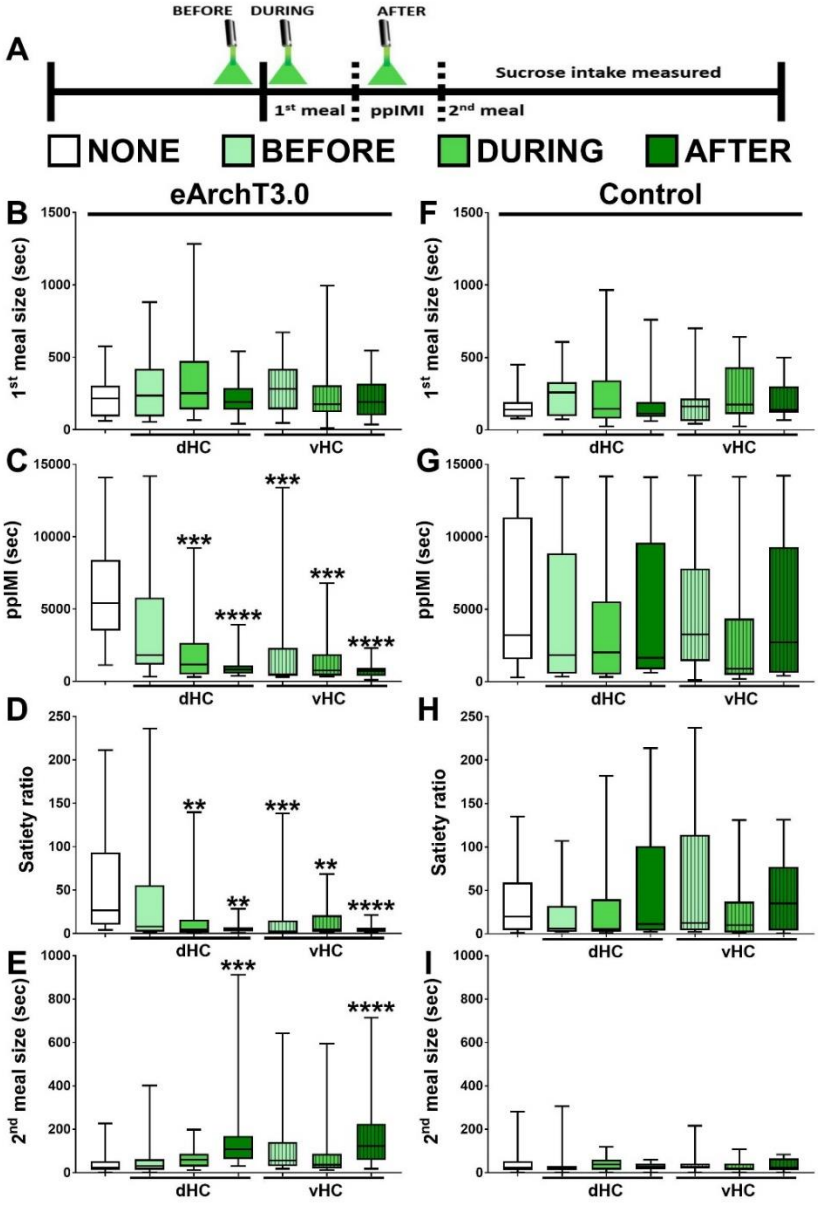

Figure 4.3: Postmeal inhibition of dHC or vHC glutamatergic neurons promoted sucrose meal initiation and increased future sucrose intake.

A*,** Time-line showing when optical inhibition of dHC or vHC glutamatergic neurons was given for 10 min relative to the first sucrose meal on different experimental days. All rats (n = 20) were given the 7 treatment conditions (i.e., within subject design) in a counterbalanced order. ***B, Optical inhibition given BEFORE, DURING, or AFTER the

first sucrose meal did not affect the size of the first meal. Optical inhibition given DURING or AFTER the first meal ***C****,* decreased the ppIMI and ***D,*** satiety ratio whereas only inhibition AFTER the first meal ***E,*** increased the amount eaten during the second meal, even though the neurons were no longer inactivated during intake of that second meal. Inhibition of vHC glutamatergic neurons given BEFORE intake of the first meal decreased the ppIMI and satiety ratio, but did not affect the other measures. In the no opsin control rats (n = 11; within-subject), illumination given BEFORE, DURING, or AFTER the first sucrose chow meal did not affect ***F***, the size of the first meal ***G***, the ppIMI, ***H,*** the satiety ratio or ***I,*** the size of the second meal. The central line depicts the median and the whiskers represent the maximum-minimum data points for each condition. ***p*<0.005; ****p*<0.0005; *****p*<0.0001 vs. NONE.

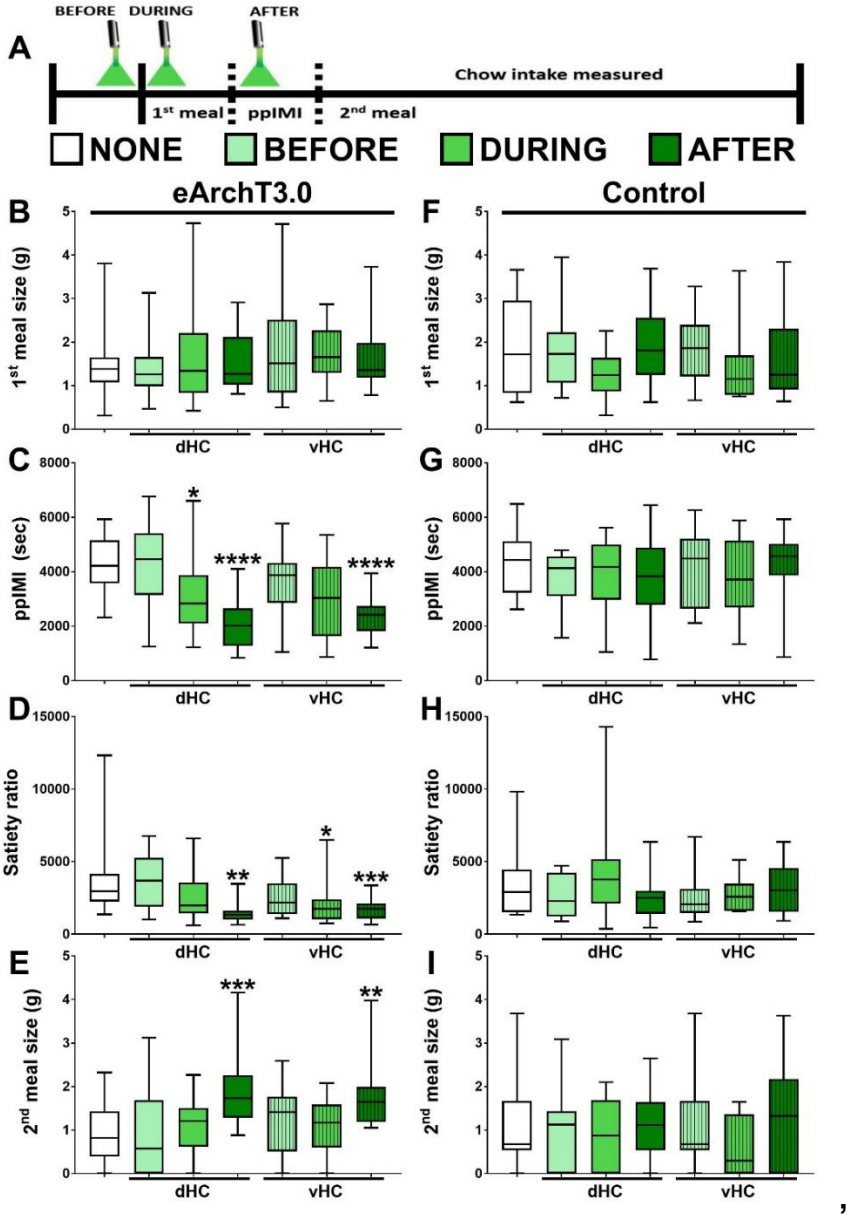

Figure 4.4: Postmeal inhibition of dHC or vHC glutamatergic neurons also promoted chow meal initiation and increased future chow intake.

***A*,** Time-line showing when optical inhibition of dHC or vHC glutamatergic neurons was given for 10 min relative to the first chow meal on different experimental days. All rats (n = 18) were given the 7 treatment conditions (i.e., within subject design) in a counterbalanced order. Optical activation of eArchT3.0 given BEFORE intake of the first

meal did not affect ***B*****,** the amount eaten during the first meal or any of the other measures; whereas, optical inhibition given DURING or AFTER the first meal ***C*****,** decreased the ppIMI and ***D*****,** satiety ratio. Only inhibition AFTER the first meal ***E*****,** increased the amount eaten during the second meal, even though the neurons were no longer inactivated during intake of that meal. In the no opsin control rats (n = 8; within-subject), illumination given BEFORE, DURING, or AFTER the first chow meal did not affect ***F***, the size of the first meal ***G***, the ppIMI, ***H,*** the satiety ratio or ***I,*** the size of the second meal. The central line depicts the median and the whiskers represent the maximum-minimum data points for each condition. ***p*<0.005; ****p*<0.0005; *****p*<0.0001 vs. NONE

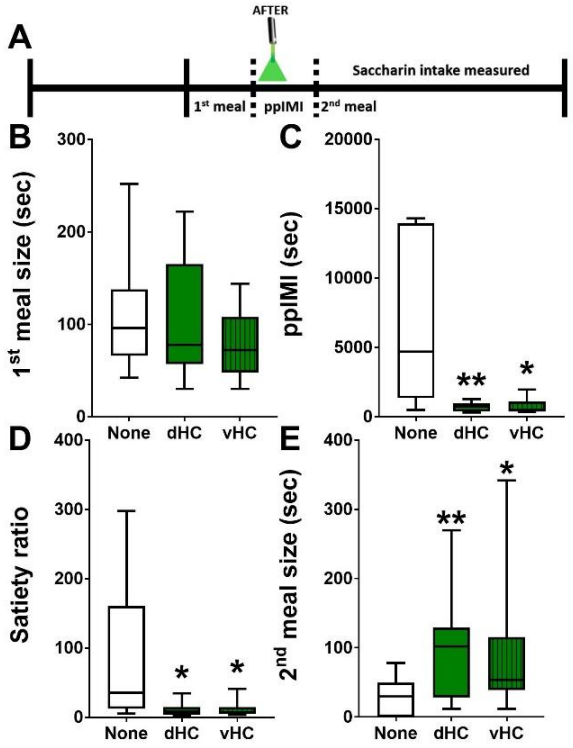

Figure 4.5: Postmeal inhibition of dHC or vHC glutamatergic neurons promoted future intake of the noncaloric sweetener saccharin.

A, Time-line showing that optical inhibition of dHC or vHC glutamatergic neurons was given for 10 min AFTER intake of the first saccharin meal. All rats (n = 13) were given both treatment conditions (i.e., NONE and AFTER; within subject design) in a counterbalanced order. Inhibition given AFTER the first saccharin meal **B,** did not affect the amount consumed during the preinhibition meal, but did **C,** decrease the ppIMI and **D,** satiety ratio and **E,** increase the amount consumed during the second saccharin meal, even though these neurons were not inhibited during intake of that second meal. The central line depicts the median and the whiskers represent the maximum-minimum data points for each condition. *p<0.05; **p<0.005 vs. NONE.

5 NMDAR-DEPENDENT SYNAPTIC PLASTICITY DOES NOT REGULATE DORSAL HIPPOCAMPAL CONTROL OF ENERGY INTAKE

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5.1 Abstract

The role of memory in regulating energy intake is not well understood. In humans, enhancing the memory of a meal reduces future intake, whereas distraction during the consumption of a meal increases feelings of hunger and increases intake during the next meal. Previous work has shown that dorsal hippocampal (dHC) neurons, which are critical for episodic memory, also inhibit energy intake during the period immediately after a sucrose meal. Also, sucrose consumption increases dHC expression of *Arc* and *ntf4*, which are known to enhance synaptic plasticity and memory formation. It is currently unknown whether dHC neurons inhibit future intake through the same process that under memory formation (i.e., synaptic plasticity). The current study tested the hypothesis that dHC neurons inhibit future intake through NMDA receptor (NMDAR)-dependent mechanisms and that dHC *Arc* and *ntf4* are critical for regulating food intake and body mass. Rats were given infusions of an NMDAR antagonist, a shRNA targeting *Arc*, a shRNA targeting *ntf4*, or their respective controls. Inhibiting dHC NMDARs or *ntf4* expression did not affect any measure of energy intake or body weight. However, 6 weeks after rats were given the shRNA targeting *Arc*, rats consumed larger-sized sucrose meals and had smaller intervals between meals compared to controls. Combined, these findings suggest that dHC neurons do not require NMDAR-dependent synaptic plasticity to inhibit future intake, but that dHC *Arc* expression may be critical for regulating intake.

Keywords: **Activity-regulated cytoskeletal protein, hippocampus, NMDARs, *ntf4***

5.2 Introduction

Most research on the neural controls of energy regulation focuses on the homeostatic and hedonic processes underlying food intake, whereas the cognitive factors regulating energy intake have been largely overlooked. A growing body of work, however, implicates the hippocampus (HC) in these processes. The HC is critical for many forms of learning and memory and can be divided into dorsal (dHC) and ventral (vHC) regions based on connectivity and function (Insausti et al. 1987, Strange et al. 2014). dHC neurons are critical for episodic and spatial memory (Shapiro et al. 2006, Hoge and Kesner 2007, Manns et al. 2007, Kesner et al. 2008, Li and Chao 2008, Quinn et al. 2008, Kennedy and Shapiro 2009, Barbosa et al. 2012), and vHC neurons are important for emotional and affective memory (Moser and Moser 1998, Bannerman et al. 2004, O'Mara et al. 2009, Barkus et al. 2010, Fanselow and Dong 2010, Kesner 2013). Most studies implicating HC neurons in energy intake focus on the role of vHC neurons (Davidson et al. 2009, Kanoski et al. 2011, Hsu et al. 2015, Hsu et al. 2015, Kanoski and Grill 2015, Hannapel et al. 2017, Hsu et al. 2017). Inhibiting vHC neurons during the period immediately after consuming a meal increases intake during the next meal and decreases the time between meals (Chapter 2; Hannapel et al. 2017). vHC neurons are also more sensitive to food-related hormonal signals than dHC neurons and have been implicated in motivational aspects of feeding (Kanoski et al. 2011, Kanoski et al. 2013, Fitzpatrick et al. 2016). Moreover, vHC neurons are the source of most HC projections to brain areas involved in feeding (i.e., lateral septum, BNST, lateral hypothalamus, and nucleus accumbens; Walaas and Fonnum 1980, Kishi et al.

2000, Cenquizca and Swanson 2006) and optogenetic excitation of these projections decreases feeding behaviors (Sweeney and Yang 2015, Hsu et al. 2017).

Evidence suggests that dHC neurons also may be critical for inhibiting energy intake. Episodic memories of a meal provide a record of intake that can likely outlast internal visceral signals generated by a meal. Patients with medial temporal lobe damage resulting in episodic memory deficits do not remember eating and will consume another meal after eating to satiety if presented with more food (Hebben et al. 1985, Rozin et al. 1998, Higgs et al. 2008). In intact humans, enhancing the episodic memory of a meal reduces intake at the next feeding bout (Robinson et al. 2013); conversely, impairing the encoding of a meal increases feelings of hunger and the amount consumed during the next meal (Brunstrom and Mitchell 2006, Higgs and Woodward 2009, Oldham-Cooper et al. 2011).

dHC neurons are also equipped to integrate food-related signals to from memories of a meal as dHC neurons express receptors for several of these signals including leptin (Mercer et al. 1996, Shioda et al. 1998), ghrelin (Xu et al. 2014), insulin (Zhao et al. 2004) and melanocortins (Gantz et al. 1993, Kishi et al. 2003, Liu et al. 2003). dHC neurons receive afferent projections from several brain regions integral to energy regulation (Tamamaki et al. 1984, Tamamaki et al. 1988, Amaral and Witter 1989, Ishizuka et al. 1990, Cui et al. 2013, Prasad and Chudasama 2013, Kondo and Witter 2014, Xu et al. 2014). Research from our lab has shown that pharmacologically inhibiting dHC neurons during the postprandial intermeal interval (i.e., the time between two meals [ppIMI]) in rats accelerates the onset of the next meal and increases intake during that meal (Henderson et al. 2013), suggesting that dHC neurons are critical for

limiting intake during the ppIMI. We have also shown that sucrose consumption increases the expression activity-regulated cytoskeletal protein (*Arc*; Bramham et al. 2010, Shepherd and Bear 2011), a master regulator of synaptic plasticity, in an experience-dependent manner in dHC neurons (Henderson et al. 2016). Moreover, sucrose consumption increases phosphorylation of the serine 831 residue ($pSer^{831}$) on the GluA1 subunit of dHC AMPA receptors (AMPARs), which is a marker of long-term potentiation (Ross et al. 2018). It is unknown, however, whether this synaptic plasticity is required for dHC regulation of energy intake.

Synaptic plasticity at excitatory glutamatergic synapses in HC is assumed to be one of the principal mechanisms of memory formation (Bailey et al. 2015, Bartsch and Wulff 2015). Enhancing synaptic strength at synapses within the HC increases functional connectivity with downstream brain regions (Canals et al. 2009) and provides a mechanism by which dHC neurons can influence feeding behavior. One of the most common forms of synaptic plasticity in dHC neurons relies on the activation of N-methyl-D-aspartate receptors (NMDARs; Bengtson and Bading 2012, Xia and Storm 2012, Kutlu and Gould 2016). NMDAR activation elevates intracellular Ca^{++} that leads to a series of molecular cascades that increase protein translation and transcribes new mRNAs, such as *Arc*, in order to enhance AMPAR function and overall synaptic strength (Davis et al. 1992, Cammarota et al. 2000, Bast et al. 2005, Bevilaqua et al. 2005, Bloomer et al. 2008, Bourne et al. 2013). Notably, pharmacologically inhibiting NMDARs or *Arc* impairs dHC-dependent memory (Guzowski et al. 2000, Guzowski et al. 2006, Czerniawski et al. 2011, Czerniawski et al. 2012, Morris 2013, Warburton et al. 2013).

In addition to the sucrose-induced increases in HC *Arc* previously observed by our lab (Henderson et al. 2016, Hannapel et al. 2017), we conducted a pilot study to determine whether sucrose consumption increased the expression of 84 genes associated with HC synaptic plasticity. This study showed that sucrose consumption increased the expression of the *ntf4* gene that encodes neurotrophic factor 4 (NT-4) nearly ~15 fold in dHC neurons. *Ntf4* increases phosphorylation of AMPARs and enhances HC synaptic plasticity (Zeng et al. 2010), and some forms of dHC-dependent memory increase *ntf4* in dHC (Callaghan and Kelly 2013). It is unknown if *ntf4* contributes to dHC inhibition of energy intake.

We hypothesize that dHC neurons form an episodic-like memory during the ppIMI that requires NMDAR dependent synaptic plasticity to inhibit future intake. If dHC neurons regulate energy intake through NMDAR-dependent synaptic plasticity, then pharmacologically inhibiting dHC NMDARs should decrease the ppIMI and increase food intake. Moreover, if *Arc* or *ntf4* are critical for dHC regulation of energy intake then using RNA interference (RNAi) to chronically knock down *Arc* or *ntf4* gene expression for months (Klein et al. 1998, Ortiz et al. 2014) should increase energy intake and body mass.

5.3 Materials and Methods

5.3.1 *Subjects*

Adult male Sprague-Dawley rats (postnatal day 52–58 on arrival; Charles River Laboratories, Wilmington, MA) were individually housed in Optirat® cages (Animal Care Systems, Centennial, CO). The rats were placed on a 12:12 light/dark cycle and

given *ad libitum* access to pelleted food and water in their home cages. The Georgia State University Institutional Animal Care and Use Committee approved all procedures.

5.3.2 *Stereotaxic Surgery*

At least 7 days after arrival, the rats were anesthetized with 5% isoflurane (Henry Schein Impromed, Oshkosh, WI) in 1,000 ml/min of oxygen (Airgas, Radnor, PA) and given penicillin (1,500 IU, im; Henry Schein Impromed, Oshkosh, WI) and carprofen (2.5 mg/kg, sc; Henry Schein Impromed, Oshkosh, WI). Anesthesia was maintained with 1–3% isoflurane gas in 500 ml/min oxygen for the duration of the surgery. Unilateral guide cannulae (3.8 mm long, 22-gauge; Plastics One, Roanoke, VA) were implanted aimed at the left or right dHC in a counterbalanced manner (AP: −3.8 mm, ML: ±2.8 mm, DV: −2.9 mm from skull (Paxinos and Watson 2007). Previous research targeting dHC with these coordinates has implicated dHC in memory and energy intake (Degroot and Parent 2000, Degroot and Parent 2001, Krebs and Parent 2005, Krebs-Kraft et al. 2007, Henderson et al. 2013). Guide cannulae were held in place by jewelers' screws (Plastics One, Roanoke, VA) and cranioplastic cement (DuraLay, Reliance Dental Mfg. Co., Worth, IL) and an obdurator (Plastics One, Roanoke, VA) was inserted into the cannula. The rats were given 0.9% sterile saline (3.0 cc, sc; Hospira, Lake Forest, IL) at the end of surgery and allowed to recover for at least 1 week before behavioral testing.

5.3.3 *Experiment 1 – Criterion based sucrose exposure*

To determine whether dHC NMDARs limit intake during the postprandial period, rats (n = 8) were given exposure to a 32% (w/v) sucrose solution at a specific time and place daily to minimize the contributions of novelty, time and contextual processes to sucrose intake. The rats were exposed to the sucrose solution daily until they consumed

the sucrose in less than 30 sec from initial presentation of the sucrose bottle for 3 consecutive days. Sucrose was used as the meal because 1) it is very palatable/rewarding to rats (Hajnal et al. 2004, Smith 2004), 2) its stimulus qualities are more specific than meals that include fats and proteins, 3) it cannot be hoarded, and 4) dHC neurons control sucrose intake during the ppIMI and sucrose consumption increases dHC *Arc* expression (Henderson et al. 2013, Henderson et al. 2016) .

On the first exposure day, the rats (n = 8) were brought to the testing room at the beginning of the light cycle, placed into polycarbonate testing cages (22 cm x 43 cm x 22 cm) that did not contain any chow but did have water, and were then given the sucrose solution 8 hr later for 10 min. Each subsequent exposure day continued in this manner with the exception that sucrose was given after 3 hr rather than 8 hr. The 8-hr interval was used initially to increase the likelihood that the rats would approach the bottle and then decreased to 3 hr to be within the range of an average ppIMI (Snowdon 1969).

5.3.3.1 Testing days and infusions

Testing days occurred 24 hr after the last sucrose exposure day. The rats were placed in their experimental cages in the behavioral testing room without food for 2.75 hr and then removed from the cages and given an intra-dHC infusion of vehicle (0.5 µl; phosphate-buffered saline, [PBS]; 0.25 µL/min, Cellgro, Manassas, VA) or D-APV (30 mM; Tocris). The injection needle extended 1.2 mm beyond the bottom of the guide cannula and was left in place for 2 min following the injection to facilitate diffusion. The rats were then returned to the experimental cage and 15 min later the rats were given sucrose for 4 hr during which meal size and the interval between meals were recorded.

This dose of D-APV was selected because it impairs NMDAR-dependent memory when infused into dHC prior to training in a memory task, but not when given afterward (Bast et al. 2005, McHugh et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Inglis et al. 2013). We reasoned that if dHC neurons are forming a NMDAR-dependent meal-related memory when they consume a meal, then it is necessary to inhibit dHC NMDARs prior to food consumption. A within-subject design was used wherein rats were given infusions of vehicle or D-APV in a counterbalanced order with 72 hr between infusions.

All intake data were recorded using a modified lickometer system that measured the change in system resistance when a rat licked from a sipper tube (Model 86062, Lafayette Instruments). The Activity Wheel Monitoring Program (Lafayette Instruments) recorded all sipper tube contacts. A sipper tube contact was operationally defined as any direct oral contact with the sipper tube longer than 3 sec (Thaw et al. 1998). This criterion improved scoring reliability by virtually eliminating all sniffs as contacts. A meal was defined as any bout containing at least 30 licks (Smith 2000, Hannapel et al. 2015). All sipper tube contacts were assumed to result in ingestion and the amount consumed was estimated indirectly by summing the duration of all sipper tube contacts during the meal. A meal was operationally defined as 5 consecutive minutes without any sipper tube contact. This criterion was used because after 5 min without consumption there is a very low probability that a rat will initiate eating again (Zorrilla et al. 2005, Fekete et al. 2007) and an increased probability of grooming, sniffing, rearing and resting behavior known as the behavioral satiety sequence (Antin et al. 1975, Thaw et al. 1998, Zorrilla et al. 2005).

Meal size was estimated by measuring the total amount of time spent in contact with the sipper tube during the meal, which excluded time spent not licking. Rats that did not consume more than one meal were given a maximum ppIMI of 14400 sec (i.e., 4 hr). To control for the effects of variations in the size of the preinfusion meal on the duration of the subsequent ppIMI, the satiety ratio was also calculated to estimate the ppIMI duration. The satiety ratio is an index of the amount of time spent not eating that is produced by the previous meal (Panksepp 1973, Zorrilla et al. 2005). It was calculated in the present experiment by dividing the ppIMI by the size of the preinfusion meal in seconds. Total sucrose intake was estimated by weighing the bottle before and at the end of the experimental session.

5.3.4 *Experiment 2 – Limited sucrose exposure*

Recent evidence from our lab showed that repeated sucrose exposure decreases *Arc* expression and $pSer^{831}$ on GluA1 subunits of AMPARs in dHC neurons (Henderson et al. 2016, Ross et al. 2018). Also, repeated training reduces dHC involvement in memory tasks (Packard and McGaugh 1996) and reduces *Arc* expression in spatial water maze (Guzowski et al. 2001). In order to minimize the possibility that sucrose overexposure reduces dHC NMDAR involvement in control of sucrose intake, rats (n = 18) underwent the same surgical procedures as in Experiment 1, but were given more limited (3 days) of sucrose exposure before the first experimental testing day. On testing days, rats were given either PBS or D-APV in a counterbalanced order in the same manner as in Experiment 1. To further determine whether the necessity of dHC NMDARs depended on the amount of sucrose exposure, the same rats were given an additional 4 days of sucrose exposure (experimental days

also provided 2 days of sucrose exposure) and then given infusions of PBS or D-APV in a counterbalanced manner on the 10th and 11th day of sucrose exposure. If overexposure to sucrose reduces the involvement of dHC NMDARs in regulating sucrose intake, then blocking dHC NMDARs should increase intake when rats were given limited sucrose exposure but not when they were given extensive exposure.

5.3.4.1 Histology – Experiments 1 and 2

After the completion of the behavioral tests, the rats were deeply anesthetized using 5% isoflurane gas (Henry Schein Impromed, Oshkosh, WI) in 1,000 ml/min of oxygen (Airgas, Atlanta, GA), decapitated, and their brains removed and stored in formalin for at least 48 hr. Brains were then sectioned (50 µm) and stained with thionin. Two observers examined the sections to estimate the infusion location 1.2 mm below the bottom of the cannula tract. In those instances when the two estimates did not agree, a third opinion was used.

5.3.5 *Experiment 3 – Chronic dHC ntf4 inhibition*

5.3.5.1 Stereotaxic surgery

Using the same surgical procedures as in Experiments 1 and 2, rats were given bilateral dHC infusions of a commercially available lentiviral construct containing either a shRNA targeting *ntf4* or a scrambled control (TL709774V, Origene; 1 µl per hemisphere; 0.125 µL/min). To allow the virus to transfect dHC neurons, the rats were allowed to recover for 2 weeks before behavioral testing.

5.3.5.2 Body Mass and Food Intake

The rats were weighed on the day of arrival and 7 days later. Rats in the control (scrambled shRNA) group and experimental group (*ntf4* shRNA) were matched on

percent change in body mass and absolute body mass. Once per week for 7 weeks, the rats were weighed and their food and water intake in their home cages were measured. The experimenters removed the rats from their home cages, weighed each animal, and then returned them to a new clean cage. Chow intake was recorded by weighing the amount of chow present in each rat's cage during the cage change and rats were provided with fresh food each week. To determine whether *ntf4* down-regulation altered sucrose intake or body mass or sucrose intake by increasing thirst, water bottles were weighed to determine total water intake.

5.3.5.3 Sucrose Exposure

Pilot studies showed that 2 weeks of viral expression was sufficient to ensure knockdown of *ntf4* and that knockdown blocked sucrose-induced increases in dHC *ntf4* expression (Figure 1A). As a result, 2 weeks after surgery the rats were given 3 days of sucrose exposure in the same manner described in Experiment 2. Behavioral testing began 24 hr after the third sucrose exposure. Rats were placed in the experimental cages for 3 hr and were then given access to the sucrose solution for 4 hr before being returned to their home cages. The rats were given sucrose exposure in this manner for 3 consecutive testing days. After the third testing day, the rats were returned to their home cages and body mass and food intake were measured for an additional 4 weeks. Then, the rats were given an additional 3 sucrose testing days (~6 weeks postsurgery). The size of the first and second meals and the intervals between meals were recorded as described in Experiments 1 and 2.

5.3.5.4 Quantitative real-time PCR (qt-PCR)

Twenty-four hours after the last sucrose testing day, the rats were anesthetized in a plastic gas induction chamber with 5% isoflurane gas (Henry Schein Impromed) in 1000 mL/min of oxygen (Airgas) until they lost their righting reflex (<1 min). The rats were rapidly decapitated using a guillotine and their brains were harvested, flash frozen in chilled 2-methylbutane (Thermo Fisher Scientific), and then stored at −80°C. The brains were sectioned (50 µm) using a cryostat (CM3050 S; Leica Biosystems) and placed on glass microscope slides. Bilateral dHC tissue punches (0.5 mm; Leica Biosystems) were taken and processed for qt-PCR.

RNA was isolated and purified using mirCURY RNA isolation kit (Exiqon). RNA concentrations were determined using a NanoDrop-2000 spectrophotometer (ND-2000, Thermo Fisher Scientific). Total RNA was reverse transcribed using Transcriptor First Stand cDNA synthesis Kit (Roche) and stored at -20 ^{o}C overnight. qtPCR was performed with commercially available primers for the housekeeping gene GAPDH (PPR06557B-200, Qiagen) and for *ntf4* (PPR49717A-200, Qiagen) using a FastStart Essential DNA Green Master Mix (Roche). Samples were run in triplicate per gene in a LightCycler 96 Instrument (Roche). The samples were pre-incubated for 10 min at 95 ^{o}C and run through 55 cycles of 3-step amplification consisting of 95 ^{o}C for 10 sec, 60 ^{o}C for 10 sec, and then 72 ^{o}C for 10 sec. Relative quantification of *ntf4* was determined using the Pfaffl method (Livak and Schmittgen 2001).

5.3.6 *Experiment 4 – Chronic dHC Arc inhibition*

To determine whether dHC *Arc* expression was critical for long-term regulation of energy intake, rats (n = 24) underwent surgery as in Experiment 3 with the exception

that they the rats were given either shRNA targeting *Arc* or the respective scrambled control (TL710356V, Origene). A pilot study confirmed that 2 weeks of virus expression was sufficient to reduce dHC *Arc* expression (Figure 1B). Rats underwent the same body mass, food intake and sucrose training procedures as described in Experiment 3 during the following 7 weeks. The qtPCR was performed with commercially available primers for the housekeeping gene GAPDH (PPR06557B-200, Qiagen) and for *Arc* (PPR44661A-200, Qiagen).

5.3.7 *Statistical Analyses*

All dependent variables were analyzed for normality using a Shapiro-Wilkes test. Outliers were identified using the GraphPad Prism ROUT method (Q = 1%). If a measure was identified as an outlier, all of the data from that rat were removed before analyzing that measure. Using this method, no outliers were removed from Experiment 1. Several outliers were removed from each data set in Experiment 2 (size of the first meal: n = 6; ppIMI: 8; satiety ratio: 11; size of the second meal: 9). In Experiment 3, three outliers were removed (satiety ratio: n = 2; size of the second meal: n = 1) and several outliers were removed from Experiment 4 (size of the first meal: n = 4; ppIMI: n = 4; satiety ratio: n = 5; size of the second meal: n = 1).

In Experiment 1, the size of the first meal was normally distributed and was analyzed using a paired student's t-test. The remaining dependent measures were not normally distributed and thus Wilcoxon signed-rank tests were used to compare the effects of vehicle and D-APV infusions on ppIMI duration, satiety ratio and the size of the second meal. For Experiment 2, the dependent measures were analyzed using a 2-way repeated measures ANOVA for drug conditions and sucrose exposure (i.e.,

minimal exposure [3-4 days] or extensive exposure [10-11 days]). For Experiment 3 and 4, all measures were analyzed using 2x2 mixed model ANOVAs with the different viruses (Control vs anti-*ntf4* or anti-*Arc*) analyzed as the between-subjects variable and the different time points relative to surgery (2 weeks vs 6 weeks) analyzed as the within-subjects variable.

Data were first assessed for sphericity using Mauchly's test. If data were below the criterion threshold for Mauchly's test, then adjusted Greenhouse-Giesser corrected measures were used. Additional t-tests with Bonferroni corrections were used to compare group data that had significant main effects. Differences in fold change of *ntf4* and *Arc* were calculated against a theoretical value of 1 (i.e., no fold change observed). Data from rats (*ntf4*: n = 1; *Arc*: n = 2) that did not show significant decreases in *ntf4* or *Arc* were added to the controls for each respective experiment and did not affect the pattern of results. Results were considered statistically significant when α values were less than 0.05. All data were analyzed using IBM SPSS Statistics for Windows, Version 21.0 (IBM Corporation, Armonk, NY) or GraphPad Prism for Windows, Version 6.0 (GraphPad Software, La Jolla, CA).

5.4 Results

5.4.1 *Experiment 1 – Premeal dHC NMDAR inhibition does not affect meal size or the duration of the ppIMI in rats given extensive sucrose preexposure.*

Compared to vehicle infusions, premeal dHC APV infusions did not increase the size of the first sucrose meal ($t(7) = 1.253$, $p = 0.2505$; Figure 2A), duration of the ppIMI

(Z = -0.2801, *p* = 0.8438; Figure 2B), satiety ratio (Z = 0.000, *p* = 0.9999; Figure 2C), nor the size of the second meal (Z = -1.4703, *p* = 0.1563, Figure 2D).

5.4.2 *Experiment 2 – Premeal dHC NMDAR inhibition does not affect meal size or the duration of the ppIMI in rats given minimal sucrose exposure.*

There were no significant main effects of drug condition (*F* (1, 68) = 0.0069, *p* = 0.9341) or duration of sucrose exposure (*F* (1, 68) = 2.463, *p* = 0.1212) on the size of the first meal (Figure 3A), duration of the ppIMI (Figure 3B; drug condition: (*F* (1, 15) = 4.521, *p* = 0.0505); sucrose exposure duration: (*F* (1, 15) = 6.366, *p* = 0.4374)), satiety ratio (Figure 3C; drug condition: (*F* (1, 12) = 0.0033 *p* = 0.9550); sucrose exposure duration: (*F* (1, 12) = 0.0200, *p* = 0.8900)), or the size of the second meal (Figure 3D; drug condition: (*F* (1, 16) = 0.6541 *p* = 0.6541); sucrose exposure duration: (*F* (1, 16) = 0.1718, *p* = 0.1718)).

5.4.3 *Experiment 3 – Down-regulating dHC ntf4 does not affect energy homeostasis.*

Seven weeks after surgery*, ntf4* expression was significantly decreased in rats given the shRNA targeting *ntf4* compared to rats given the scrambled control (Figure 4C, Z =-231.0, p = 0.001). There were no significant differences between rats given the shRNA targeting *ntf4* and rats given the scrambled control across all measures of sucrose intake. The size of the first meal (Figure 5A, shRNA: (*F* (1, 19) = 0.63 *p* = 0.4372); time: (*F* (1, 19) = 1.34, *p* = 0.2614)), duration of the ppIMI (Figure 5B, shRNA: (*F* (1, 19) = 0.13 *p* = 0.7224); time: (*F* (1, 19) = 1.08, *p* = 0.311743)) and satiety ratio (Figure 5C, shRNA: (*F* (1, 15) = 0.02 *p* = 0.8894); time: (*F* (1, 15) = 0.34, *p* = 0.5685)) were not significantly different between the two groups. There was however a significant

main effect of time on the size of the second meal (Figure 5D, shRNA: (F (1, 18) = 3.3 p = 0.0860); time: (F (1, 18) = 11.29, p = 0.0034), indicating that rats given the control virus and the shRNA ate less during the second meal 6 weeks after surgery compared to 2 weeks after surgery. Virus type did not affect body weight or food intake, but there was a main effect of time on body weight (Figure 4A, shRNA: (F (1, 21) = 0.205 p = 0.655); time: (F (2.775, 59.267) = 330.213, p = 0.0001)) and food intake (Figure 4B, shRNA: (F (1, 21) = 0.028 p = 0.868); time: (F (3.340, 70.144) = 4.241, p = 0.0063)). Regardless of virus condition, rats weighed significantly more and consumed more food, but less water, 6 weeks after surgery compared to 2 weeks after surgery (shRNA: (F (1, 21) = 1.488 p = 0.236); Time: (F (3.435, 92.059) = 2.921, p = 0.033)).

5.4.4 *Experiment 4 – Down-regulating dHC Arc increases the size of the sucrose first meal and decreases the ppIMI 6 weeks postsurgery.*

In rats given the shRNA targeting *Arc*, baseline *Arc* expression was significantly decreased compared to rats given the scrambled control seven weeks after surgery (Figure 6A, Z =-231.0, p = 0.001). The results showed a significant main effect of virus type on the size of the first meal (Figure 7A, shRNA: (F (1, 17) =7.300 p = 0.0151); time: (F (1, 17) = 0.983, p = 0.358) and the duration of the ppIMI (Figure 7B, shRNA: (F (1, 17) =0.756, p = 0.3973); time: (F (1, 17) = 13.435, p = 0.0192). Post hoc t-tests showed that 2 weeks after shRNA infusions, there were no differences in the size of the first sucrose meal between rats given the shRNA and control rats (t(17) = 1.612, p = 0.1253). However, 6 weeks after shRNA infusions, rats given the shRNA targeting *Arc* ate larger first meals than controls (t(17)=2.361, p = 0.0304). For the duration of the ppIMI, there were no significant differences between experimental and control rats (t(17)

= 0.703, *p* =0.4915) 2 weeks after shRNA infusions. Six weeks after rats were given the shRNA targeting *Arc,* they consumed their second sucrose meal significantly sooner than controls (t(11.67) = 2.222, *p* = 0.0469). There was also a main effect of week on the satiety ratio (Figure 7C, shRNA: (*F* (1, 16) = 0.759, *p* = 0.3965); time: (*F* (1, 16) = 6.194, *p* = 0.0242) indicating that rats had lower satiety ratios at 6 weeks compared to 2 weeks. There were no main effects of virus condition or time on the size of the second meal (Figure 7D, shRNA: (*F* (1, 20) = 0.047, *p* = 0.8306); time: (*F* (1, 20) = 0.138, *p* = 0.7142). Rats given either virus condition weighed more and consumed more food 6 weeks after surgery than 2 weeks after surgery (Figure 7B, Body weight: shRNA: (*F* (1, 21) = 0.6540 *p* = 0.428); Time: (*F* (1.326, 27.844) = 238.760, *p* = 0.0001; Average weekly chow intake: (Figure 7C, shRNA: (*F* (1, 21) = 0.733 *p* = 0.402); Time: (*F* (3.540, 74.343) = 5.321, *p* = 0.0013)) Average weekly water intake did not differ between virus conditions and was not affected by time (shRNA: (*F* (1, 21) = 0.154 *p* = 0.699); Time: (*F* (4.831, 101.457) = 2.000, *p* = 0.087)).

5.5 Discussion

The studies outlined in the paper suggest that dHC NMDARs do not regulate sucrose intake, and that the extent of sucrose exposure prior to training did not affect whether dHC NMDARs influence sucrose intake. Moreover, chronic knockdown of *ntf4* did not affect sucrose consumption, intake of standard chow, or body weight. Chronic knockdown of dHC *Arc* did not affect body weight or intake of standard chow, but did affect two measures of sucrose intake. Specifically, dHC infusions of the anti-*Arc* shRNA increased the size of the first sucrose meal and decreased the ppIMI 6-week posttransfection. Although these results may seem conflicting as *Arc* is typically

expressed in an NMDA-dependent manner (Bloomer et al. 2008, Czerniawski et al. 2011) and inhibiting dHC NMDARs did not affect energy intake, these findings may inform how alternative mechanisms mediate dHC regulation of energy intake.

The lack of effects observed in both APV experiments may be the result of several factors. Although the dose of D-APV (30mM) used in this study was chosen from previous papers showing that this dose inhibits dHC-dependent memory (Bast et al. 2005, McHugh et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Inglis et al. 2013), several other studies have uses higher and lower doses of D-APV to disrupt dHC-dependent memory (Baker and Kim 2002, Misane et al. 2005, Quinn et al. 2005, Boisselier et al. 2017). These different doses of D-APV may prove more effective for disrupting dHC regulation of energy intake then the dose used in this study. It is unlikely that the negative effects of APV were due to insufficient duration of NMDAR inhibition as D-APV inhibits NMDAR function for at least 100 min (Morris 1989) and therefore should still be inhibiting dHC NMDARs during the first meal and most of the ppIMI.

Alternatively, the number of sucrose exposures given before testing may have limited our ability to observe a dHC NMDAR-dependent effect on energy regulation. In Experiment 1, rats were given daily exposure to sucrose until they consumed sucrose within 30 sec of being given access to sucrose for 3 consecutive days. Rats met this criterion after 8-12 days of sucrose exposure. This repeated exposure to sucrose may have impacted dHC involvement in regulating sucrose intake as work from our lab and others show that repeated sucrose exposure reduces dHC measures of synaptic plasticity (Henderson et al. 2016, Ross et al. 2018). Moreover, repeated training or exposure to testing environments reduces dHC involvement in memory tasks (Packard

and McGaugh 1996), reduces *Arc* expression (Guzowski et al. 2006) in spatial water maze (Guzowski et al. 2001) and lever-pressing tasks (Kelly and Deadwyler 2002, Kelly and Deadwyler 2003), and decreases HC expression of molecules essential for memory formation, such as phosphorylated cAMP response element-binding protein (pCREB; Moncada and Viola 2006) and protein kinase M-ζ (Moncada and Viola 2008). Experiment 2 was designed to test whether repeated sucrose exposure influenced the effects of dHC NMDAR blockade by reducing sucrose exposure prior to testing and by determining whether the amount of sucrose exposure interacted with the effects of blocking dHC NMDARs. The results from Experiment 2 suggest that dHC NMDARs are not involved in regulating sucrose intake regardless of degree of sucrose preexposure. The current study did not examine whether blocking dHC NMDARs would affect intake in rats given less than 3 days of sucrose exposure because our previous research determined that rats need at least 3 days of sucrose exposure to reliably consume sucrose during the testing period. Moreover, testing was not conducted on the first 2 days of sucrose exposure as dHC neurons also respond to novel stimuli (reviewed in (Lee et al. 2005). It is possible that dHC NMDARs regulate energy intake during those first 2 days of exposure, but any effects observed during those initial days could be due to dHC responses to novelty.

The results from the previously-conducted gene array study showed that sucrose consumption increased dHC *ntf4* expression and an additional pilot study showed that the anti-*ntf4* shRNA blocked sucrose-induced increases in *ntf4* (Figure 1A). The results of Experiment 3, however, showed that dHC *ntf4* does not regulate energy intake. The shRNA targeting *ntf4* significantly knocked down dHC *ntf4* expression, but there were

no functional effects on energy homeostasis. Even without a 100% knockdown of *ntf4*, the *ntf4* knockdown (~7 fold) should be sufficient to prevent its effects, as similar amounts of knockdown inhibit synaptic plasticity and memory (Green et al. 2010, Voleti et al. 2012, Zhang et al. 2015). It is possible that the sucrose-induced increase in dHC *ntf4* expression observed in the array and in our pilot work with the specific primer was due to gastric distention (Wang et al. 2006, Wang et al. 2008, Xu et al. 2008, Min et al. 2011, Spetter et al. 2014), licking or locomotive behavior. Including additional control animals given sucrose exposure as described above, but given water instead of sucrose prior to euthanasia may determine if dHC *ntf4* increases are the result of these alternate processes.

The chronic knockdown of dHC *Arc* in Experiment 4 provides mixed results regarding the role of dHC *Arc* in inhibiting future intake. When rats were tested 2 weeks after they were given the shRNAs, there were no statistically significant effects; however, at 6 weeks, rats given the shRNA targeting *Arc* consumed larger meals when first presented with sucrose, and ate their second meal sooner than rats given the scrambled control. It is also possible that the effects are due to a more effective knockdown of dHC *Arc* at 6 weeks after surgery compared to knockdown 2 weeks after surgery. Our pilot study based on a small sample size (n = 5) showed that the *Arc* shRNA decreased *Arc* expression decreased by 2.178 fold 2 weeks after surgery (Figure 1B) and by approximately 6 fold 6 weeks after surgery.

Alternatively, the significant effects observed during the second test are could be due to the recruitment of metabotropic glutamate receptors (mGluRs) caused by the repeated sucrose exposures. mGluR activation, like NMDAR activation, increases

intracellular Ca^{++} and upregulates *Arc* expression (Waung et al. 2008, Kumar et al. 2012, Wilkerson et al. 2014). Whether mGluRs are critical for the formation of new memories than NMDARs depends on familiarity. When an animal is first learning a task, such as the location of an escape platform in a spatial water maze, inhibiting NMDARs impairs learning; however, if an animal has prior experience in the task (i.e., preexposure in another water maze with a different escape platform location) inhibiting NMDARs does not affect the formation of new memories i.e., they are NMDAR-independent (Bannerman et al. 1995, Quinlan et al. 2004, Tayler et al. 2011, Wiltgen et al. 2011, Crestani et al. 2018). If mGluRs are pharmacologically inhibited after initial training in a task, then this ability to create new NMDAR-independent memories is blocked (Crestani et al. 2018). This suggests that mGluRs support memory formation when a task is familiar, but not when the task is novel. In Experiment 4, sucrose intake was tested twice, once 2 weeks after surgery and again 6 weeks later. It is possible that testing at 2-weeks acts as preexposure for the testing at 6 weeks, and that the testing at 6 weeks requires mGluR-dependent increases in *Arc* expression to inhibit energy intake. As a result, the decrease in dHC *Arc* caused by the anti-*Arc* shRNA could block the mGluR mechanism for synaptic plasticity (i.e., *Arc*) and increase the size of the first meal and decrease the ppIMI during the second test but not the first. It should be noted, however, that this type of NMDAR-independent learning dissipates over time (Crestani et al. 2018). The longer the interval is between the first and second testing block the less the likelihood that NMDAR-independent memories are formed. It is unknown whether the delay of 28 days used in the current study is longer than the interval necessary to maintain NMDAR-independent learning (Crestani et al. 2018). To

determine if mGluRs enhance dHC regulation of energy intake, additional studies inhibiting mGluRs after sucrose preexposure are needed

The lack of statistical differences between experimental and control groups throughout these experiments are not likely the result of small effect sizes and limited sample size. Post-hoc power analyses ($\alpha = 0.025$; $\beta = 0.80$) show that an unreasonable number of rats would need to be added to detect significant differences and would likely not change the main conclusions of these experiments. Depending on the specific measure, an additional 47-9811 animals would be necessary to achieve significant differences between the vehicle and APV groups for Experiment 1 and for Experiment 2, an additional 72-249 rats would be required. For Experiment 3, an additional 23-192,975 rats would need to be added to detect a significant difference between rats given the anti-*ntf4* shRNA and the scrambled control. For Experiment 4, 31-178 rats would be needed to detect additional significant effects of the anti-*Arc* shRNA compared to the scrambled control.

In summary, the findings in the current paper do not support the hypothesis that dHC NMDARs and dHC *ntf4* are critical for inhibiting energy intake. These experiments show that dHC *Arc* may be critical for inhibiting sucrose intake if rats have previous exposure to sucrose in a familiar context and raise the possibility that mGluRs are involved in that process.

5.6 Acknowledgments

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MH104384 (RTL), a GSU Center for Obesity Reversal predoctoral fellowship (RCH) and the Center for Behavioral Neuroscience.

5.7 Figures

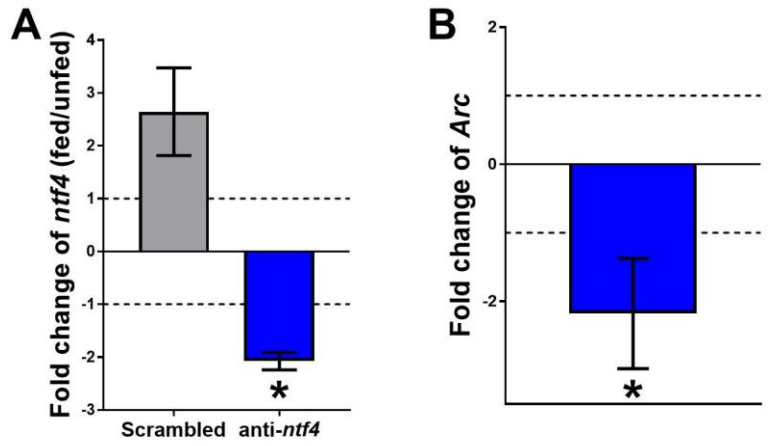

Figure 4.6*: *Pilot studies showed that sucrose consumption appears to increase ntf4 in dHC neurons 2 weeks after surgery and shRNAs effectively down-regulate dHC ntf4 and Arc.

A, Compared to cage control rats given a scrambled shRNA in dHC (n = 2), rats given scrambled shRNA in dHC and access to sucrose (n = 2) appeared to have higher dHC ntf4 expression (normalized to the housekeeping gene *gapdh),* by contrast dHC ntf4 expression was significantly reduced in rats given dHC anti-*ntf4* shRNA (n =2) compared to cage control dHC anti-*ntf4* shRNA (n = 2). ***B,*** Compared to rats given scrambled shRNA in dHC (n = 5), rats given an anti-*Arc* shRNA in dHC (n = 5) had lower baseline dHC *Arc* expression 2 weeks after surgery. Gene expression was normalized to *gapdh*. *$p < 0.05$ vs hypothetical value of 1.

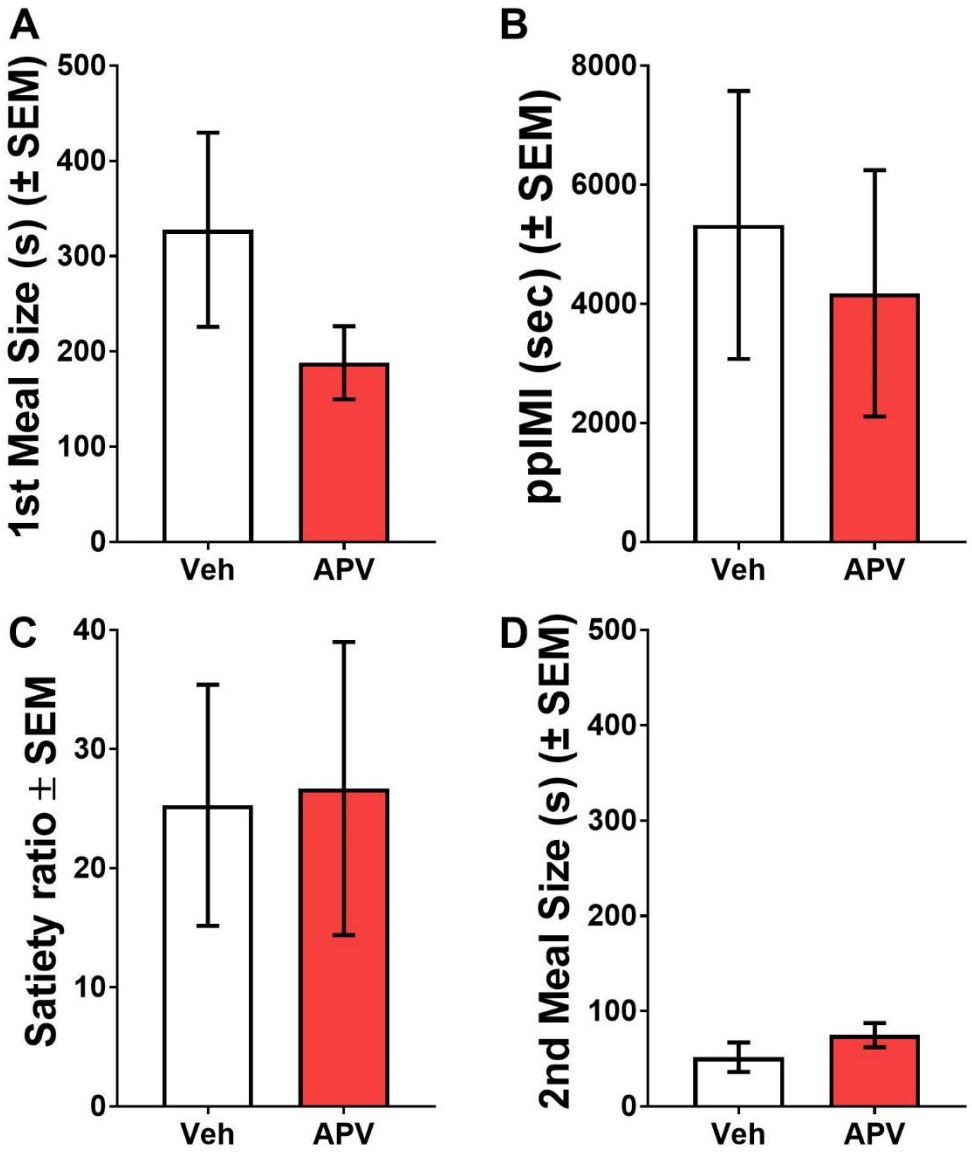

Figure 4.7: Premeal inhibition of dHC NMDARs did not affect subsequent energy intake in rats given extensive sucrose exposure prior to the injection days.

A, Compared to the effects of dHC vehicle infusions, premeal dHC APV infusions (n = 8; within-subject) did not significantly affect the size of the first meal ***B,*** the ppIMI ***C,*** satiety ratio ***D,*** or size of the second meal in rats given extensive sucrose exposure prior to the injection days..

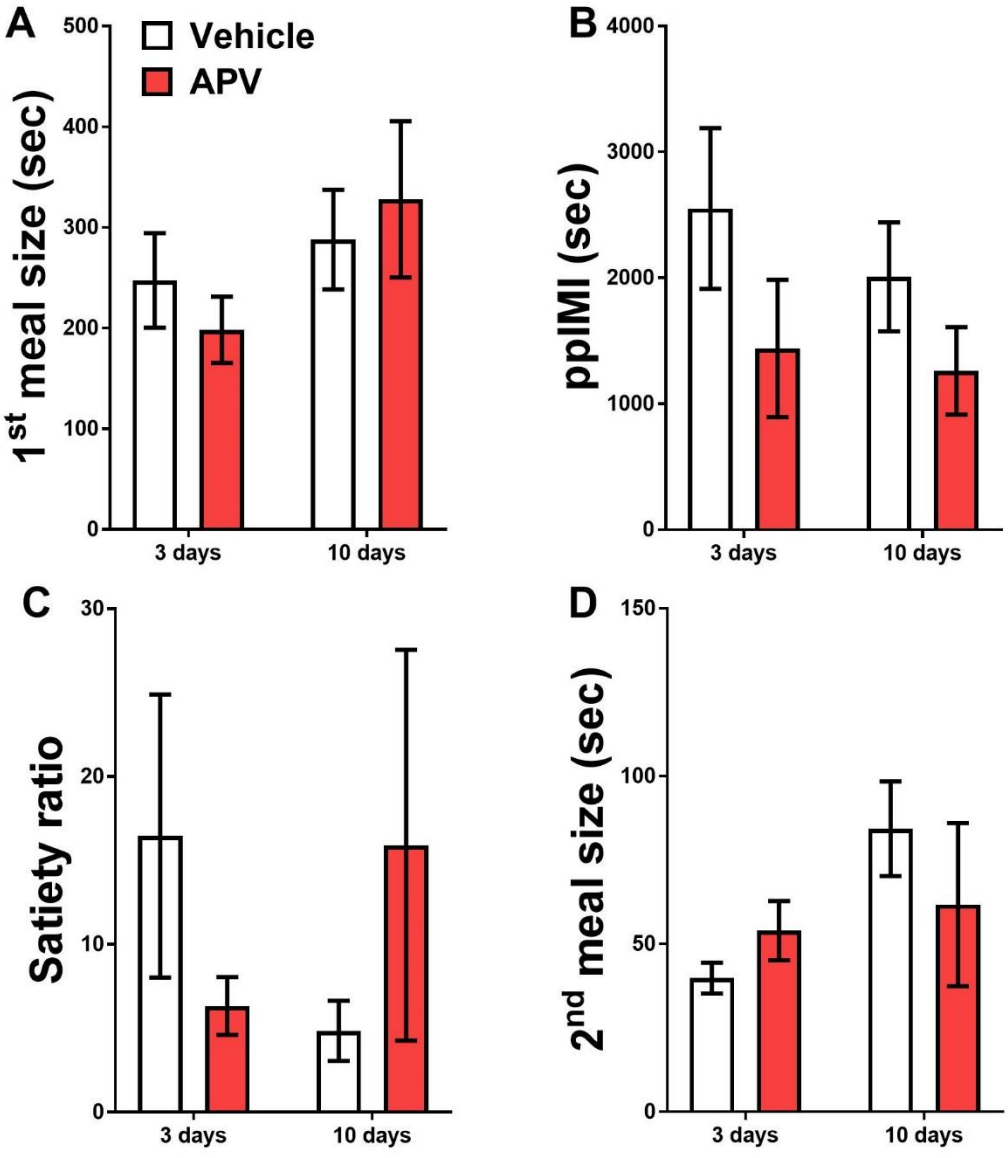

Figure 4.8: Premeal inhibition of dHC NMDARs did not affect energy intake in rats given minimal sucrose preexposure prior to the injection days.

A, Compared to dHC vehicle infusions**,** premeal dHC APV infusions (n = 18; within-subject) did not affect the size of the first meal ***B,*** the ppIMI ***C,*** satiety ratio ***D,*** or size of the second meal in rats given 3 days and then 10 days of sucrose exposure.

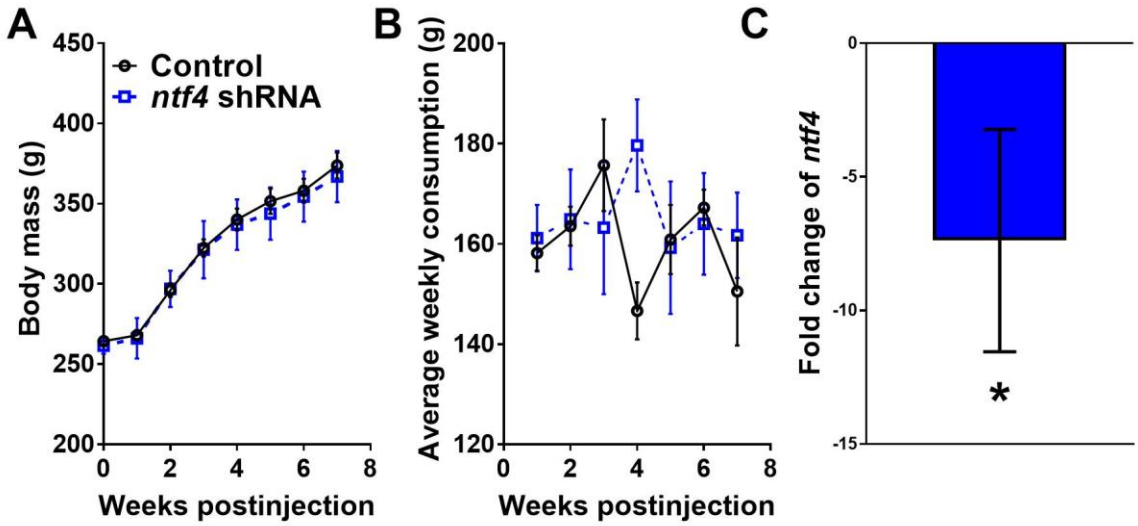

Figure 4.9: Chronic knockdown of dHC ntf4 did not affect body weight or standard chow intake, but did significantly decrease the expression of dHC ntf4. Compared to scrambled controls (n = 11), ***A,*** rats given the anti-*ntf4* shRNA (n =10) in dHC did not weigh more or ***B,*** consume more standard chow during the experimental period. ***C,*** The anti-*ntf4* shRNA did significantly knock down-regulate dHC *ntf4* expression measured 7 weeks after infusions. **p* < 0.05 vs Control

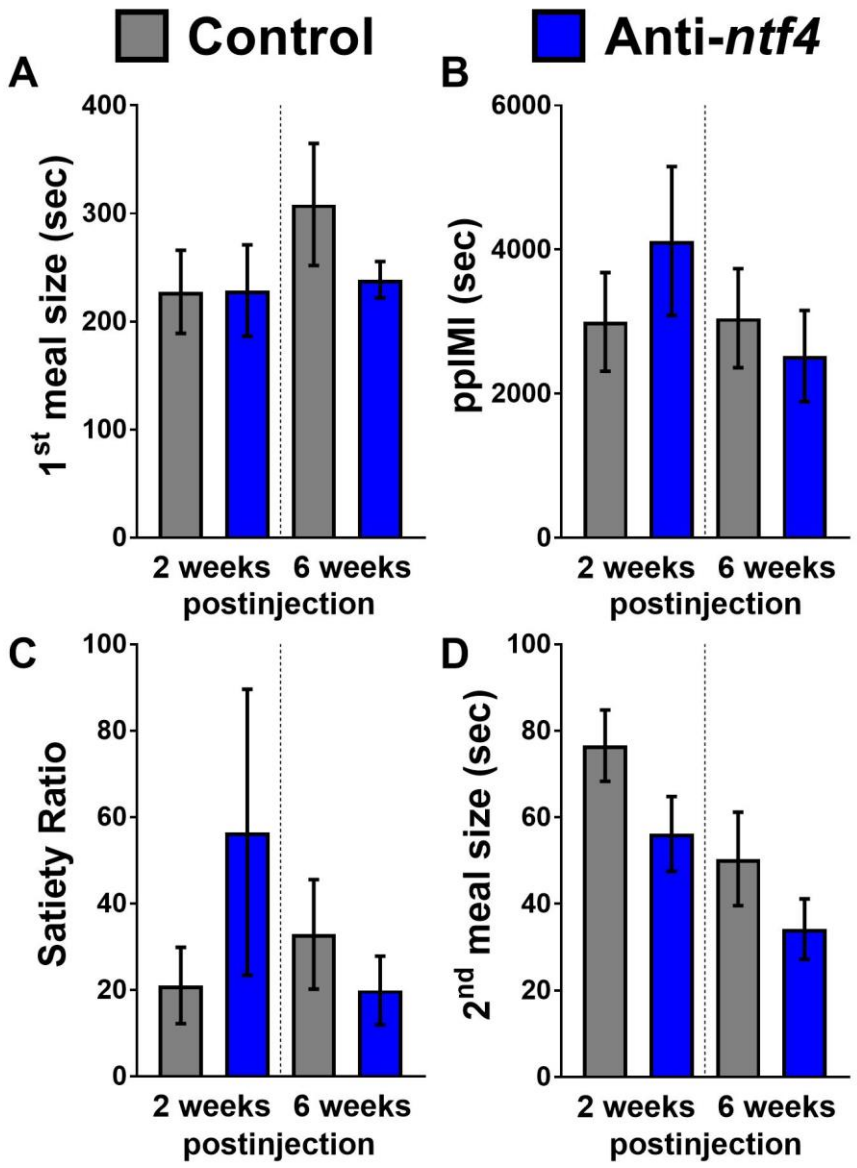

Figure 4.10: Chronic knockdown of dHC ntf4 did not affect energy intake.

Rats were given either a shRNA knocking down dHC *ntf4* (anti-*ntf4*) or a scrambled control (Control: n = 12; anti-*ntf4*: n = 11) and then given sucrose 2 and 6 weeks later. Compared to scrambled controls, the anti-*ntf4* shRNA did not significantly affect the ***A,*** size of the first meal ***B,*** the duration of the ppIMI ***C,*** satiety ratio ***D,*** or the size of the second meal.

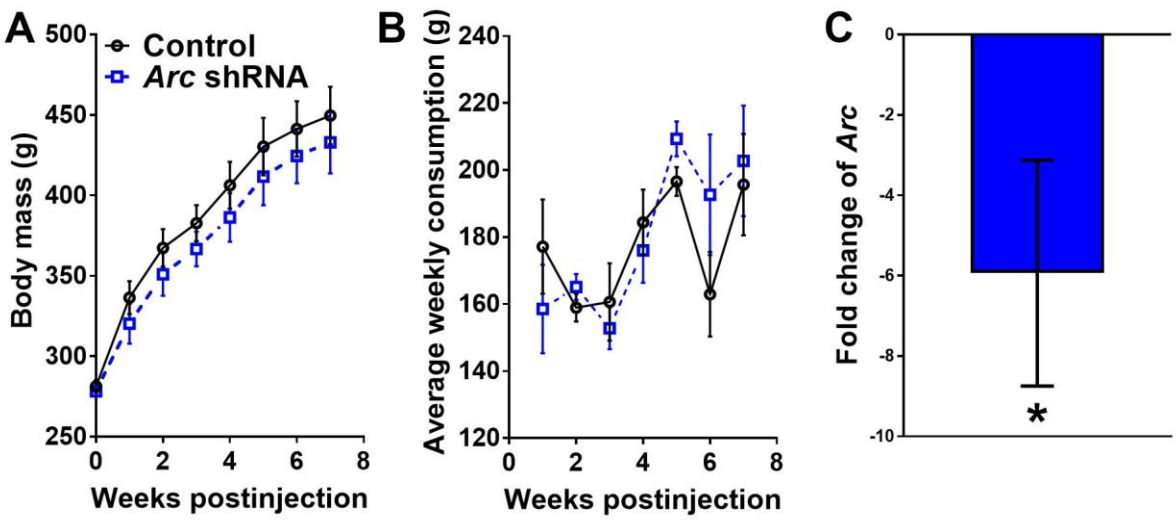

Figure 4.11*: *Chronic knockdown of dHC Arc did not affect body weight or standard chow intake, but did decrease dHC Arc expression measured 7 weeks postinjection.

A, Compared to rats given a scrambled control virus, rats given dHC anti-*Arc* shRNA did not weigh more or ***B,*** consume more standard chow during the experimental period. ***C,*** The anti-*Arc* shRNA significantly down-regulated down dHC *Arc* expression. *$p <$ 0.05 vs Control

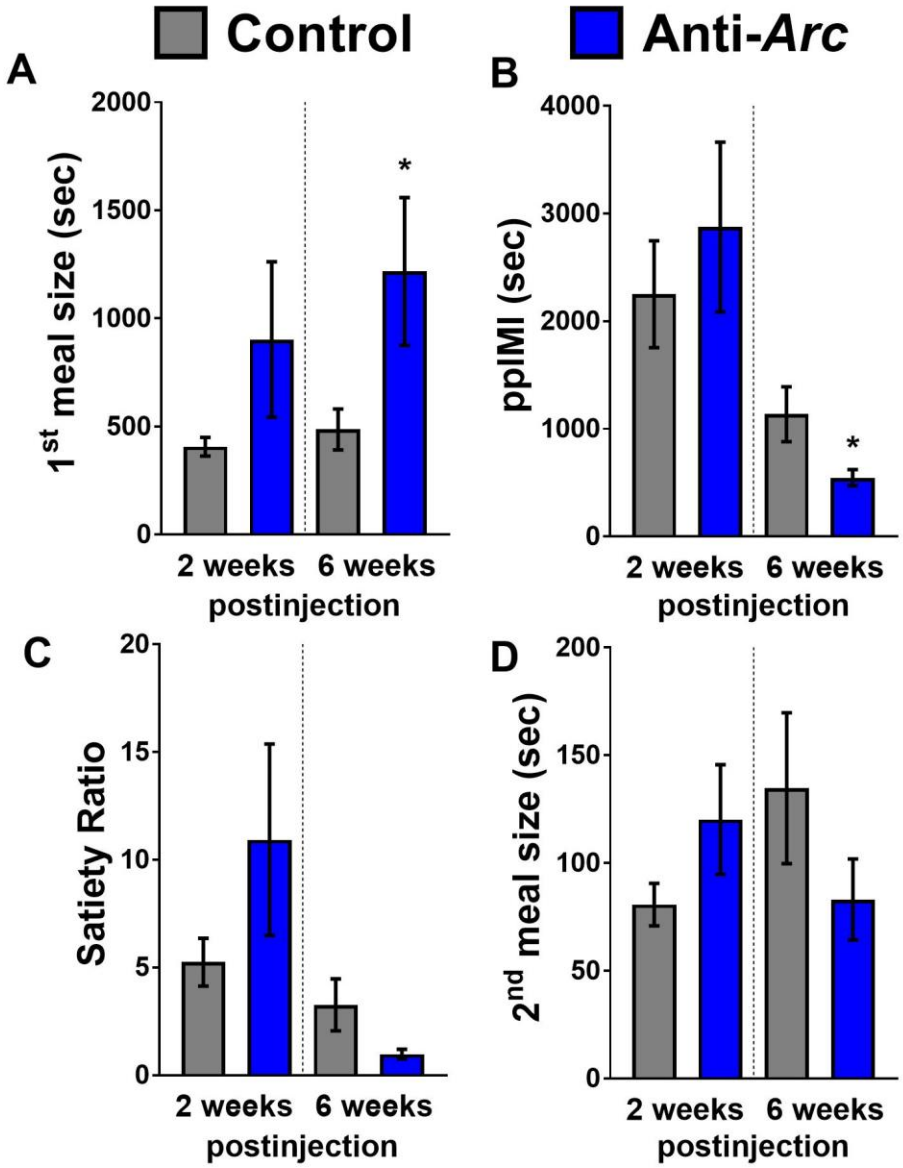

Figure 4.12: Chronic knockdown of dHC Arc increased the size of the first sucrose meal and decreased the time between the first and second meal 6 weeks after the dHC anti-Arc shRNA injections.

Rats were given dHC injections of either a shRNA to knock down *Arc* or a scrambled control (Control: n = 12; anti-*Arc*: n = 12; between subject) and then given sucrose 2 weeks and 6 weeks after infusions. Compared to the scrambled control, ***A,*** the dHC

anti-*Arc* shRNA did not increase the size of the first meal during testing at 2 weeks after the injections, but did increase the size of the first meal 6 weeks after the injections. ***B,*** In a similar manner, the anti-*Arc* shRNA did not affect the duration of the ppIMI 2 weeks after the injections, but decreased the duration of the ppIMI 6 weeks after the injections. ***C,*** Compared to the scrambled control, the anti-*Arc* shRNA did not affect the satiety ratio ***D,*** or size of the second meal at either time-point. *$p < 0.05$ vs Control

6 DISCUSSION

6.1 General summary

The overarching goal of this dissertation was to determine whether HC neurons inhibit energy intake through mechanisms that also underlie learning and memory. To test this hypothesis, we asked 1) whether vHC neurons inhibit energy intake during the ppIMI and whether this process required NMDAR activation and vHC Arc expression, 2) whether dHC and vHC principal glutamatergic neurons are critical for inhibiting energy intake during the postprandial period when the memory of a meal would be undergoing consolidation and 3) whether dHC NMDARS, *Arc* and *ntf4* are required for dHC inhibition of energy intake. The current findings show that both dHC and vHC neurons inhibit energy intake during the ppIMI and suggest that vHC neurons inhibit intake through mechanisms requiring NMDAR-dependent synaptic plasticity. Specifically, this dissertation is the first to show that pharmacologically inhibiting vHC neural activity or blocking vHC NMDARs decreases the ppIMI and increases sucrose consumption during the next meal (Hannapel et al. 2017; Chapter 3); optogenetically inhibiting dHC or vHC neurons immediately after a sucrose, chow, or saccharin meal decreases the ppIMI and increases intake during the next meal (Chapter 4). Inhibiting dHC NMDARs or chronic knockdown of dHC *Arc* or *ntf4* expression does not affect standard chow intake or body weight; however, chronic knockdown of dHC *Arc* expression may increase some aspects of sucrose intake.

The studies outlined in this dissertation show that some of the underlying mechanisms required for memory formation are also required for regulating energy intake and support the hypothesis that HC neurons form meal-related memories during

the ppIMI and inhibit future intake. Collectively, the findings within this dissertation show that dHC and vHC neural activity is critical for limiting energy intake during the time when meal-related memories should be encoded and consolidated. Additionally, disrupting mechanisms underlying memory formation in vHC neurons increases intake and decreases the time between meals. These findings suggest that understanding the cognitive controls of energy intake such as memory can provide new pathways by which to investigate energy regulation disorders.

6.2 AIM 1: vHC neurons inhibit energy intake during the ppIMI and limit intake through NMDAR-dependent mechanisms

The first study (Hannapel et al. 2017) outlined within this dissertation shows that temporary inactivation of vHC activity following the consumption of a sucrose meal decreases the ppIMI and increases intake during the next meal. It also shows that sucrose consumption increases *Arc* expression, a master regulator of synaptic plasticity (Bramham et al. 2010, Shepherd and Bear 2011), in vHC neurons (Hannapel et al. 2017). These findings are consistent with studies showing that HC neurons influence eating behavior and body weight (Davidson et al. 2009, Parent et al. 2014, Kanoski and Grill 2015). vHC ghrelin signaling increases feeding behavior (Kanoski et al. 2013, Hsu et al. 2015), whereas vHC GLP-1 (Hsu et al. 2015) or leptin decreases feeding (Kanoski et al. 2011). Additionally, continuous excitation of vHC glutamatergic projection neurons during a 30-min session of food access reduces food intake (Sweeney and Yang 2015). These studies, however, did not restrict their manipulations in relation to meal timing (i.e., during a meal or immediately after a meal). Previous evidence from our lab has shown that inhibiting dHC neurons specifically after the completion of a meal also

decreases the ppIMI and increases intake during the next consumed meal (Henderson et al. 2013) and sucrose consumption increases *Arc* expression in dHC neurons albeit to a greater degree than in vHC neurons (Henderson et al. 2016). The larger increase of *Arc* expression in dHC than vHC neurons is consistent with previous work that shows higher levels of synaptic plasticity in dHC neurons compared to vHC neurons (Papatheodoropoulos and Kostopoulos 2000, Papatheodoropoulos and Kostopoulos 2000, Maruki et al. 2001, Maggio and Segal 2007), although it is still unclear whether dHC synaptic plasticity contributes to energy regulation. The increases in energy intake found after temporarily inhibiting vHC neurons specifically after the consumption of a meal (i.e., during the time when a meal-related memory should be undergoing consolidation) shows that postprandial vHC neural activity is necessary for inhibiting future intake. It is likely that postprandial signals such as leptin that increase synaptic plasticity in HC (Shanley et al. 2001, Harvey et al. 2006) and enhance vHC memory processing (Kanoski et al. 2011) also contribute to the formation of meal-related memories during the postprandial period.

Consolidation of HC-dependent memory typically requires neural activity and NMDAR-mediated synaptic plasticity at glutamatergic synapses (Luscher and Malenka 2012, Bailey et al. 2015). Specifically, NMDAR-dependent *Arc* expression is required for most dHC- and vHC-dependent memory (Guzowski et al. 2000, Guzowski et al. 2001, Guzowski 2002, Bramham et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Chia and Otto 2013). Pharmacologically inhibiting HC NMDARs inhibits synaptic plasticity and disrupts memory formation (Czerniawski et al. 2011, Czerniawski et al. 2012, Park et al. 2014). Therefore, if vHC neurons are forming meal-related memories,

then disrupting mechanisms necessary for memory formation within vHC neurons should also increase energy intake. Chapter 3 shows that inhibiting either vHC NMDARs or vHC *Arc* expression during the consumption of the first meal increases the amount consumed during the first meal and reduces the satiety ratio, indicating that rats ate their next meal sooner relative to the amount of food they consumed during their first meal (i.e., the rats were less satiated; Zorrilla et al. 2005). These findings were of particular note as impaired encoding of a meal in intact humans does not affect the size of the meal currently being eaten but increases the amount consumed during the next meal and decreases feelings of satiety (Brunstrom and Mitchell 2006, Oldham-Cooper et al. 2011).

It is possible that blocking vHC NMDARs and disrupting vHC *Arc* expression does not affect the acquisition and encoding phase of a meal-related memory, but interferes with ongoing memory consolidation, which in turn leads to a longer first meal and smaller satiety ratio. Once a memory-inducing event has occurred, processes underlying both encoding and consolidation begin. The manipulations used in Chapter 3 targeted mechanisms of consolidation, not acquisition. NMDARs are required for memory consolidation, but are not necessary for memory acquisition (Burgos-Robles et al. 2007, Liu et al. 2014). Moreover, inhibiting *Arc* expression does not affect the induction of LTP or memory acquisition on HC-dependent tasks, but impairs the maintenance of LTP and disrupts long-term memory consolidation (Guzowski et al. 2000). This suggests that the increases in sucrose consumption during the first meal were due to disrupted memory consolidation, not impaired acquisition. Large increases in meal size are also observed in patients with severe hippocampal damage. After

eating to satiation, these patients will consume additional food if presented with it, increasing the amount consumed at that sitting (Hebben et al. 1985, Rozin et al. 1998, Higgs 2008).

6.3 AIM 2: dHC and vHC glutamatergic neurons are critical for inhibiting future energy intake during the period after the consumption of a meal.

The findings from Chapter 2 and 3 show that inhibiting vHC activity after a meal or vHC NDMARs during a meal increases energy intake. These studies disrupted vHC function using pharmacological manipulations (i.e., muscimol, APV, or *Arc* antisense, respectively) that remain effective for hours (Morris 1989, Martin 1991, Arikan et al. 2002, Guzowski 2002), and likely lasted throughout the ppIMI and during the consumption of the next meal. For the findings in Chapter 2, it is impossible to determine whether the increases in the size of the second meal are a result of inhibition during the ppIMI or due to continued vHC inhibition during intake of the second meal. Likewise, it is impossible to determine whether the decreases in the size of the satiety ratio in Chapter 3 are the result of processes that were impaired during the first consumed meal or impaired processes during the ppIMI. These issues limit the potential interpretation of when HC neurons are necessary for regulating future intake.

To determine when dHC and vHC glutamatergic neurons are most critical for inhibiting future intake, we restricted optogenetic inhibition of dHC or vHC glutamatergic neurons to the period before, during, or after a sucrose, chow, or saccharin meal. Specifically inhibiting dHC or vHC neurons during or after the first sucrose meal decreases the ppIMI, whereas only dHC or vHC inhibition given after the first meal increases the amount consumed during the second meal. Additionally, inhibition of dHC

or vHC neurons given after the first chow or saccharin meal decreases the ppIMI and increases the size of the second meal. These data show that inhibition of dHC or vHC neurons during the consumption of a meal does not increase future intake as strongly as when inhibition is applied following the consumption of a meal. This may be the result of the postmeal inhibition disrupting more of the consolidation period than inhibition during the consumption of meal. This interpretation is supported in part by the findings from Chapter 2 and 3 showing that postmeal infusions decrease the ppIMI and increase the size of the meal, whereas premeal blockade of vHC NMDARs only increased intake during the first meal. Interestingly, optogenetic inhibition of vHC neurons during the first meal did not increase the size of that meal nor did it increase the size of the second meal. This suggests that vHC NMDAR-dependent mechanisms are required for limiting the size of the first meal, whereas impairing vHC neural activity during that meal does not affect the size of the first meal. Together, these findings suggest that the critical period for meal-related dHC and vHC neuronal activity extends past the initial consumption of a meal and into the ppIMI, when a meal-related memory should be consolidated. These findings support the hypothesis that consolidation of meal-related memories inhibit future intake and suggests that the consolidation period for a meal-related memory includes the time during which a meal is consumed.

It is possible that postmeal optogenetic inhibition of dHC or vHC neurons interferes with visceral postprandial signaling, thus leading to increases in future intake. HC damage in humans impairs the ability to interpret interoceptive signals such as hunger and pain (Hebben et al. 1985, Rozin et al. 1998, Higgs et al. 2008). In rodents, HC lesions impair the ability to discriminate interoceptive cues of food deprivation or

satiation (Davidson and Jarrard 1993, Davidson et al. 2010). For example, rats trained to approach a food magazine containing sucrose pellets after either no food deprivation or 24-hr of food deprivation had increased food-seeking behaviors regardless of the their hunger state following HC-lesions (Davidson et al. 2010). Moreover, inhibiting postprandial signals such as leptin or GLP-1 signaling in vHC neurons also increases energy intake and inhibits food-related memories (Kanoski et al. 2011, Hsu et al. 2015). If visceral postprandial signaling is required for HC regulation of intake, then inhibiting dHC or vHC neurons during the postprandial period should not affect intake of the non-caloric sweetener saccharin, as it produces minimal visceral postingestive consequences (Mook et al. 1980, Renwick 1985, Sclafani and Nissenbaum 1985, Renwick 1986). Sucrose and chow intake are partially controlled by gastrointestinal and postabsorptive mechanisms (Strader and Woods 2005, Cummings and Overduin 2007), whereas saccharin intake is primarily controlled by orosensation rather than interoceptive, postingestive signals (Byard and Goldberg 1973, Mook et al. 1980, Mook et al. 1981, Kushner and Mook 1984, Renwick 1985, Sclafani and Nissenbaum 1985, Renwick 1986). The findings of this dissertation show that inhibiting dHC or vHC neurons after a saccharin meal increases intake during the next meal and decreases the ppIMI, suggesting that HC neurons do not require visceral postprandial signals to inhibit future intake, thereby supporting the memory consolidation hypothesis.

The findings of this dissertation suggest that sweet taste alone is sufficient to induce a meal-related memory that inhibits future intake because inactivating dHC or vHC neurons after a saccharin meal when a meal-related memory should be undergoing consolidation increases intake (Chapter 4) and saccharin intake increases

dHC (Henderson et al. 2013) and vHC *Arc* expression (Hannapel et al. 2017). Saccharin consumption increases *Arc* expression in dHC neurons to a greater degree than intake of an isopreferred concentration of sucrose suggesting that consumption of saccharin induces stronger meal-related memories than sucrose consumption (Henderson et al. 2016). It is unknown whether saccharin intake increases *Arc* expression in vHC neurons. Alternatively, saccharin-induced increases of dHC *Arc* expression may not be the result of a meal-related memory as hypothesized with sucrose and chow consumption, but a separate learning experience wherein rats learns to dissociate the sweet-taste from caloric load (Swithers et al. 2012). Essentially, with saccharin consumption, rats learn to no longer associate sweet-taste with food that produces caloric intake and instead learn that sweet-tasting food is less satiating. Inhibiting dHC or vHC neurons after a saccharin meal may impair the ability to discriminate between sweet taste and caloric content causing rats to consume more saccharin during the second meal than when dHC and vHC neurons are uninhibited. This interpretation is supported by evidence showing that the ingestion of saccharin-sweetened foods leads to overconsumption of similar foods as rats no longer associate sweet taste with the caloric content (Swithers 2015). To dissociate between whether the postmeal optogenetic inhibition-induced increased saccharin intake is due to impaired meal-related memory versus impaired knowledge of sweet taste and caloric content, one could excite dHC or vHC neurons during the postprandial period. After repeated trials, rats would either continue to ingest less saccharin or wait longer to consume saccharin if the stimulation was enhancing meal-related memories, whereas if the

excitation increased the association between saccharin and absence of caloric load, then rats should consume more saccharin during their next meal.

6.4 AIM 3: dHC NMDARs and *ntf4* are not required for inhibiting future intake and chronic knockdown of dHC *Arc* expression may inhibit long-term sucrose intake.

Ntf4 appeared to be a promising candidate for controlling dHC regulation of energy intake. A pilot study from our lab showed that sucrose consumption increased the expression of *ntf4* ~15 fold in dHC neurons and decreased it by ~4 fold in vHC neurons. This was the largest increase of any plasticity-related gene in the 84-gene array. Additionally, some forms of HC-dependent memory increase *ntf4* (Callaghan and Kelly 2013) and *ntf4* increases AMPA receptor phosphorylation and enhances HC synaptic plasticity (Zeng et al. 2010). If dHC *ntf4* also enhances meal-related memories, then knocking down dHC *ntf4* should increase energy intake. The present findings show, however, that long-term knockdown of dHC *ntf4* does not increase sucrose intake, body weight or chow consumption during the experimental period. This is particularly surprising as transgenic deletion of the *ntf4* gene inhibits long-term memory (Fan et al. 2000, Xie et al. 2000). The present findings suggest that dHC *ntf4* is not critical for regulating energy intake. It is possible that the large increases in *ntf4* observed in the array study were induced from gastric distention (Wang et al. 2006, Wang et al. 2008, Xu et al. 2008, Min et al. 2011, Spetter et al. 2014), licking or locomotive behavior during sucrose exposure. To determine whether licking behaviors and ingestion of liquids also increase *ntf4*, rats would need to be given sucrose

exposure but on testing days be given water instead of sucrose to ensure the rat approaches the bottle and licks as during a regular sucrose exposure.

Although sucrose consumption increases measures of synaptic plasticity in dHC neurons, these particular forms of plasticity do not appear to be necessary for dHC regulation of energy intake. Synaptic plasticity should lead to the strengthening of a meal-related memory that delays the onset of the next meal. Sucrose consumption increases *Arc* expression and markers of long-term potentiation in dHC neurons (Henderson et al. 2016, Ross et al. 2018). However, the results of this dissertation show that pharmacologically inhibiting dHC NMDARs or dHC *Arc* has limited effects on energy intake. Specifically, inhibiting dHC NMDARs does not affect intake when rats are given repeated exposure to sucrose prior to testing (Chapter 5). This finding alone could be partially explained due to an attenuation of HC involvement due to repeated exposure (Packard and McGaugh 1996) because increasing the number of training trials rats are given in a behavior task decreases dHC expression of molecules critical for memory, including *Arc* (Kelly and Deadwyler 2002, Kelly and Deadwyler 2003, Moncada and Viola 2006, Moncada and Viola 2008). However, this dissertation also showed that inhibiting dHC NMDARs did not affect sucrose consumption in rats given minimal sucrose exposures (i.e., 3 days). Rats were not given less than 3 days of exposure because that was the fewest number of exposures needed to have rats reliably consume sucrose during the 4-hr testing period. dHC neurons in particular respond to novel stimuli (reviewed in Lee et al. 2005) and blocking dHC NMDARs on the first day of sucrose exposure may impair novelty detection instead of HC-dependent memory. There are several possible explanations for the lack of effects of inhibiting dHC

NMDARs on energy intake. It is possible that 3 days of sucrose exposure reduces NMDAR-dependent synaptic plasticity in dHC neurons. This is unlikely as the sucrose induced increases in dHC synaptic plasticity previously observed (Henderson et al. 2016, Ross et al. 2018) occurred with the same amount of sucrose exposure or more than the current study. It is also possible that sucrose-induced increases in dHC synaptic plasticity are statistically different than their respective controls, but not functionally important because sucrose-induced dHC *Arc* expression is smaller than levels observed in other learning tests (Nalloor et al. 2012, Nalloor et al. 2014). It is also possible that the inhibition of dHC NMDARs was ineffective at altering sucrose intake due to the drug dosage used in the study. The dose of D-APV (30mM) used to inhibit NMDARs was chosen from previous papers showing that this dose inhibits dHC-dependent memory (Bast et al. 2005, McHugh et al. 2008, Czerniawski et al. 2011, Czerniawski et al. 2012, Inglis et al. 2013); alternatively, several other studies have used higher and lower doses of D-APV to disrupt dHC-dependent memory (Baker and Kim 2002, Misane et al. 2005, Quinn et al. 2005, Boisselier et al. 2017). The current findings suggest that dHC neurons do not require NMDAR-dependent synaptic plasticity to inhibit future intake, even though inhibiting dHC neural activity after a meal increases intake (Henderson et al. 2013; Chapter 4). This also suggests that dHC neurons do not form a meal-related memories as memory formation requires synaptic plasticity (Takeuchi et al. 2014, Bailey et al. 2015, Bartsch and Wulff 2015). The interpretation that dHC neurons do not require synaptic plasticity to regulate energy intake is further supported by the evidence that 2 weeks after dHC *Arc* knockdown rats do not consume

more sucrose, weigh the same, and show no differences in home cage food consumption compared to control animals (Chapter 5).

It is possible that dHC neurons regulate energy intake through NMDAR-independent synaptic plasticity. This interpretation is supported by the findings 6 weeks after dHC *Arc* knockdown when the rats were tested for a second time. During this second testing block, rats consume larger first meals and have shorter ppIMIs than controls. The increases in the size of the first meal and decreases in the duration of the ppIMI are particularly surprising as the more frequently the rats are exposed to sucrose the less HC *Arc* should be involved (Guzowski et al. 2006, Henderson et al. 2016). Inhibiting NMDARs blocks *Arc* expression in HC-dependent memory tasks (Czerniawski et al. 2011, Czerniawski et al. 2012). *Arc* expression, however, can also be induced by mGluR activation, which increases intracellular Ca^{++} (Waung et al. 2008, Kumar et al. 2012, Wilkerson et al. 2014). It is possible that the increases in the size of the first meal and decreases ppIMI observed at 6 weeks are due to the recruitment of mGluRs instead of NMDARs when rats are tested for a second time, and potentially provide an explanation for the discrepancy between the findings at observed at 2 weeks and at 6 weeks. NMDARs are essential for the initial learning of a task such as spatial water maze, but inhibiting NMDARs does not affect the formation of new memories when a rat has had prior experience in the task (e.g., changing the platform location in the water maze; Bannerman et al. 1995, Quinlan et al. 2004, Tayler et al. 2011, Wiltgen et al. 2011, Crestani et al. 2018). Specifically, rats trained to find an escape platform in a water maze using extramaze cues particular to a room located downstairs were also able to learn the location of an escape platform in a room located upstairs with different

extramaze cues. However, when dHC NMDARs were inhibited during the second learning event in the room located upstairs, rats given previous training in the room located downstairs learned the location of the escape platform faster than rats not given training in the room located downstairs (Bannerman et al. 1995). These findings suggest that some HC-dependent learning may be NMDAR-independent (Bannerman et al. 1995). If, however, mGluRs are inhibited during a subsequent learning event such as finding the location of the escape platform in the room located upstairs, rats are unable to acquire this new information, suggesting that NMDAR-independent memories require mGluR activation (Crestani et al. 2018). It should be noted that during the *Arc* shRNA experiment, NMDARs were not inhibited during the first or second sucrose testing blocks. Therefore, it is possible that the increases in the size of the first meal and decreases in the duration of ppIMI observed at 6 weeks when the rats were tested for a second time were due to inhibiting dHC *Arc* expression that would be mGluR-mediated. Additional studies would be needed to determine if dHC mGluRs regulate energy intake. Determining whether inhibition of dHC mGluRs would increase intake in rats given multiple sucrose testing experiences would help elucidate the role of dHC mGluRs in feeding behavior. This does not explain, however, why the anti-*Arc* shRNA did not affect intake at 2 weeks. One possibility is that other NMDARs-dependent mechanisms of synaptic plasticity, such as the extracellular signal-regulated kinase (ERK) pathway that are critical for memory and do not require *Arc* signaling to influence synaptic plasticity, influence dHC energy intake (reviewed in Peng et al. 2010, but see Waltereit et al. 2001). Alternatively, it is possible that dHC *Arc* was not sufficiently down-regulated 2 weeks after surgery, but was 6 week after surgery. Together, the findings from the dHC

Arc knockdowns suggest that dHC *Arc* may inhibit the intake of previously encountered energy sources. Moreover, this suggests that factors other than dHC NMDARs, such as dHC mGluRs, may be involved in dHC regulation of energy intake.

6.5 Do dHC and vHC have functionally distinct roles in regulating energy intake?

The major findings in this dissertation lead to several new questions. Both dHC and vHC neurons inhibit energy intake during the ppIMI, but this does not mean dHC and vHC serve identical roles in regulating feeding behavior and that underlying mechanisms for this inhibition are the same. Neural activity in dHC neurons immediately following a meal delays the onset of the next meal and limits intake during the next meal, but it does not appear that *Arc* expression or NMDAR-dependent synaptic plasticity is necessary for this inhibition (Henderson et al. 2013; Chapter 4 and 5). In contrast, neural activity in vHC neurons during the consumption of meal or immediately after a meal delays the onset of the next meal and this process requires NMDAR-dependent synaptic plasticity and *Arc* expression to inhibit future intake (Hannapel et al. 2017; Chapter 3 and 4). dHC and vHC neurons, however, are functionally distinct (Fanselow and Dong 2010). dHC neurons encode episodic memories that represent what, where, and when something occurred (Shapiro et al. 2006, Hoge and Kesner 2007, Manns et al. 2007, Kesner et al. 2008, Li and Chao 2008, Quinn et al. 2008, Kennedy and Shapiro 2009, Barbosa et al. 2012), whereas vHC neurons are important for motivation, affect and emotional memory (Moser and Moser 1998, Bannerman et al. 2004, O'Mara et al. 2009, Barkus et al. 2010, Fanselow and Dong 2010, Kesner 2013).

It is possible that dHC neurons do not form a meal-related memory, but rather support the formation of vHC mediated meal-related memories. dHC neurons are

connected with vHC neurons through longitudinal projections (Amaral and Witter 1989, Yang et al. 2014), and activation of dHC glutamatergic neurons increases neural activity in vHC neurons, but vHC activation does not affect dHC neurons (Takata et al. 2015). vHC neurons, in turn, are the primary source of HC projections to downstream brain regions involved in feeding behavior (Kanoski and Grill 2015). Feeding behavior increased measures of synaptic plasticity in dHC and vHC neurons, whereas only inhibiting vHC NMDAR-dependent synaptic plasticity increases energy intake (Hannapel et al. 2017; Chapter 3). Indeed, increased HC synaptic strength enhances functional connectivity between HC neurons and downstream brain regions (Canals et al. 2009), providing a mechanism for synaptic plasticity in vHC neurons to influence intake. In support of this, optogenetic excitation of vHC glutamatergic projections to the medial prefrontal cortex, lateral septum, or bed nucleus of the stria terminalis decrease feeding behavior (Sweeney and Yang 2015, Hsu et al. 2017). It is possible that dHC activity immediately following a meal enhances vHC output to downstream brain regions, and that inhibiting dHC neurons decreases vHC signaling, thereby reducing vHC output to downstream regions and increasing food consumption.

A related possibility is that inhibition of dHC neurons during the period immediately following a meal disrupts HC theta rhythms and diminishes vHC regulation of intake. In rats, dHC rhythmic oscillations of neural activity within a range of 4-10 Hz (Hasselmo 2005, Munn et al. 2015) are referred to as theta waves and are strongly associated with dHC memory (Hasselmo 2005, Buzsaki and Moser 2013, Jacobs 2014). Increased HC theta rhythms correlate with measures of learning and memory (Berry and Thompson 1978, Winson 1978, Seager et al. 2002), whereas manipulations that

decrease HC theta rhythms (Rawlins et al. 1979) are associated with HC-dependent memory impairments (Martin et al. 2007). Additionally, dHC theta rhythms are entrained to food availability (Munn et al. 2015) and propagate through the HC along the longitudinal axis from dHC through vHC (Patel et al. 2012). To test whether HC theta rhythms influence energy intake, one could record HC activity before, during, and after the consumption of meal and determine whether consumption affects HC theta rhythms and whether interfering with HC theta rhythms impairs energy intake. If interfering with dHC theta rhythms increases energy intake, then this could account for the similar effects of dHC and vHC inhibition and for the finding that vHC inhibition produced stronger effects than dHC inhibition.

6.6 Do dHC neurons influence energy intake through non-mnemonic mechanisms?

It is possible that inhibiting dHC neurons interferes with the ability of dHC neurons to track elapsed time. Some dHC neurons show robust activity that corresponds with the passing of time (Itskov et al. 2011, MacDonald et al. 2011). These "time cells" contribute to organizing the temporal order of events and help distinguish between similar experiences (Wood et al. 2000, Pastalkova et al. 2008, MacDonald et al. 2011). Patients with medial temporal lobe damage have impaired ability to estimate time (Richards 1973, Noulhiane et al. 2007), and lesions specific to dHC neurons produce similar deficits in rodents (Tam and Bonardi 2012, Tam and Bonardi 2012). Inhibiting dHC neurons with muscimol degrades the ability of dHC neurons to discriminate the timing between events (Jacobs et al. 2013). These impairments lead to an underestimation of elapsed time, or the perception that the interval between events

is shorter. The decrease in the ppIMI observed when dHC neurons are inhibited after intake may be therefore caused, in part, by impaired perception of elapsed time.

It is possible that optogenetically or pharmacologically inhibiting dHC neurons disrupts the circadian cycle and increases energy intake. Rodents fed at regular intervals show increases in neural activity before food availability (Edmonds 1977, Escobar et al. 2009). Evidence also suggests that dHC CA1 neurons may be part of a food-entrainable oscillator (Munn and Bilkey 2012) outside of the main suprachiasmatic nucleus oscillators (Escobar et al. 2009) that influences circadian cycles. These food-entrainable oscillators can override rhythmicity imposed by the light/dark cycle, particularly in rodents given restricted food access (Herrero et al. 2005), or given scheduled feeding (Angeles-Castellanos et al. 2011). Although none of the studies in this dissertation restricted food access, rats were given sucrose at the same time and place each day. This scheduled access to sucrose may have entrained dHC activity to sucrose availability and as a result inhibiting dHC neurons may have caused a leftward shift in circadian cycles, decreasing the time between meals. In humans and rodents, meals consumed later in the circadian cycle tend to be larger and contribute to increased body fat (Clifton et al. 1984, Arble et al. 2009, Scheer et al. 2013, McHill et al. 2017). If inhibiting dHC neurons during the ppIMI accelerates the circadian cycle, then rats may consume larger meals following inhibition simply due to an altered circadian cycle.

6.7 Limitations

A significant limitation of this dissertation is that we were unable to show whether dHC or vHC neurons form a meal-related memory. Previous work has shown that it is

possible to measures an animal's ability to remember where it consumed food (Rubinow et al. 2009), or which arm of a maze food is available in (McDonald and White 1993), but not if the memory of a specific meal drives behavior. Modern neuroscience techniques can use a combination of genetic and viral interventions that selectively target and "tag" ensembles of neurons that are activated by a learning-event. Researchers can use optogenetic constructs that inhibit or excite these tagged neurons to block memory or induce a memory (Liu et al. 2012, Ramirez et al. 2013, Ramirez et al. 2015). The targeting of neurons specific to a memory experience allows researchers to determine whether the memory formed during an event drives behavioral changes as opposed to simply HC activity. Although it is hypothesized in this dissertation that HC neurons form-meal related memories that inhibit future intake, it is impossible to determine whether the memory of meal inhibits future intake without specifically manipulating HC-neurons activated by an eating event. For the purposes of this dissertation, if HC neurons that were activated during meal consumption were able to be tagged with an inhibitory optogenetic construct (Liu et al. 2012, Ramirez et al. 2013, Ramirez et al. 2015), it would then be possible to determine whether subsequent inhibition of those neurons would accelerate the onset of the next meal or increase intake during the next meal. This could help determine whether HC neurons form-meal related memories

Inhibition of dHC and vHC neurons shows that these brain regions are necessary to limit intake, but does not show whether activation of these neurons is sufficient to suppress future intake. Limited evidence shows that optogenetic excitation of vHC projections to the medial-prefrontal cortex, LS, or BNST (Sweeney and Yang 2015, Hsu

et al. 2017) decreases total food intake, but this excitation was not limited to the period during or after consumption. To determine whether dHC and vHC neurons are sufficient to delay meal onset and limit future intake, it would be necessary tag HC neurons activated during meal consumption with an excitatory optogenetic construct (Ramirez et al. 2013, Ramirez et al. 2015) and drive activity in those tagged neurons to potentially delay the onset of the next meal or decrease intake during the next meal.

It is impossible to directly compare the two studies that manipulated *Arc* expression in vHC (Chapter 3) and dHC neurons (Chapter 5) due to methodological differences. The vHC *Arc* manipulation was acute (*Arc* antisense) and the dHC manipulation was chronic (*Arc* shRNA). The vHC *Arc* antisense experiment used a within-subject design (n = 9), whereas the chronic knockdown of *Arc* using the shRNA used a between-subjects design (Controls: n = 12; Arc shRNA: n = 12). It is therefore possible that the *Arc* shRNA study is underpowered to detect a difference between the experimental and control group. This is unlikely, however, as sucrose consumption increases *Arc* expression in dHC neurons (Henderson et al. 2013) more than in vHC neurons (Hannapel et al. 2017) and the *Arc* shRNA knockdown was greater in dHC neurons (~6.5 fold) than the *Arc* antisense knockdown in vHC neurons (~2.5 fold). This leaves two possibilities, 1) dHC *Arc* expression is not critical for inhibiting future intake, or 2) the effectiveness of dHC *Arc* in inhibiting future intake is much less than in vHC neurons and a study would need significantly more subjects than the number of subjects used in the current work to detect any role for dHC *Arc* in regulating energy intake.

It remains unknown whether other mechanisms of synaptic plasticity are involved in energy intake and whether dHC neurons utilize different mechanisms of synaptic plasticity than vHC neurons. The current studies selectively targeted *Arc* because it is considered to be a master regulator of synaptic plasticity (Bramham et al. 2010, Shepherd and Bear 2011), and sucrose consumption increased *Arc* expression in dHC and vHC neurons. The pilot gene array study that identified *ntf4* as a possible target for manipulation, however, found that sucrose consumption increased 20 other genes more than 1.5 fold in dHC neurons. These 20 genes provide promising targets for future manipulation, such as the inflammatory cytokine TNF-α, which increased ~7 fold by acute sucrose intake in dHC neurons. It unknown exactly how proinfammatory cytokines may contribute to HC regulation of energy intake, but expression of proinflammatory cytokines such as TNF-α is increased in obese patients and can enhance energy expenditure (Ye and McGuinness 2013, Wang and Ye 2015), increase the expression of the anorexigenic leptin receptor (Gan et al. 2012), yet impair HC-dependent memory (Golan et al. 2004, Beilharz et al. 2014, Ohgidani et al. 2016). The gene array only tested the effects of sucrose consumption on synaptic plasticity-related genes and did not determine whether consumption of non-sweetened foods or non-caloric sweeteners would have a similar effect on the pattern of gene expression. Consumption of saccharin increases *Arc* expression in dHC neurons more so than sucrose consumption (Henderson et al. 2016). It is therefore possible that dHC neurons do form meal-related memories as consumption of non-sweetened foods or non-caloric sweeteners could increase the expression of other genes critical for synaptic plasticity not tested within this dissertation.

6.8 Conclusion

The data presented in this dissertation show that impairing the function of dHC and vHC neurons critical for memory, at specific times when these neurons are likely to be necessary for memory consolidation, decreases the time between meals and increases future intake. Moreover, disrupting mechanisms essential to memory consolidation in vHC neurons increases future intake. Collectively, these findings suggest that HC neurons inhibit energy intake and that synaptic plasticity in vHC neurons controls meal timing and meal size.

These findings fill a gap in our understanding of neural mechanisms that inhibit meal initiation and meal frequency. These findings are particularly important now as more than one-third of the adult population in the United States is now considered overweight or obese (Ogden et al. 2013, Ogden et al. 2014, Ogden et al. 2014). Excessive intake of calories from palatable foods and beverages are significant factors contributing to the development and maintenance of obesity (Cutler et al. 2003, Bleich et al. 2008, Swinburn et al. 2009, Mozaffarian et al. 2011, Monteiro et al. 2013, Moubarac et al. 2013, Slining et al. 2013). In humans, being overweight or obese is associated with hippocampal atrophy (Cherbuin et al. 2015) and episodic memory deficits (Cheke et al. 2016). Similarly in rats, excess intake of fats and/or sugars impairs HC synaptic plasticity (Grillo et al. 2011, Karimi et al. 2013) and HC-dependent memory (Ross et al. 2009, Ross et al. 2012, Darling et al. 2013). HC lesions (Clifton et al. 1998, Davidson et al. 2009) or temporarily inactivating dHC or vHC neurons increases meal frequency and food intake (Henderson et al. 2013, Hannapel et al. 2017). Obesity may be, in part, caused and maintained by this vicious cycle of HC damage leading to

increased intake (Davidson et al. 2005, Davidson et al. 2014, Hargrave et al. 2016). Understanding how cognitive factors such as enhancing meal-related memory can reduce intake may provide new, more effective interventions for limiting intake and promoting weight loss (Robinson et al. 2013, Robinson et al. 2014).

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